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From: LeGuyader, John
Sent: Thursday, February 18, 1999 10:16 AM
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Human Mol. Genet. 1(9):769-71, 1992.

Nucleic Acids Res. 19(24):6982 to end of article, 1991.

Investigative Opthalmology and Visual Science 36(4):ps1045, 1995, please attach date of availablity of this volume and number of journal.

Investigative Opthalmology and Visaul Science 36(1):62-71, 1995, please attach date of availablity of this volume and number of journal.

American Journal of Human genetics 55 (3 Suppl.):pA358, 1994.

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Gene Therapy 1 (Suppl. 1):pS61, 1994.

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Polymorphism in the protein C gene detected by denaturing gradient gel electrophoresis

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Paris V, Paris, France

Description: Exon VIII of the protein C gene (1) was amplified using 5'-(G/C)$_{50}$-ATATGAAACCAGGTGCCCT-3' and 5'-T-CCCCCTACCCAGGCCTCTG-3' as the 5' and 3' primers respectively. The upstream primer had added at its 5'-end a 50 nucleotide-G + C rich region designed with a computer program, in order to create a higher melting temperature domain. This allowed the analysis of a domain comprising the whole exon and its exon/intron junctions by Denaturing Gradient Gel Electrophoresis (DGGE).

This amplified 296 bp fragment corresponds to nucleotides 7063 to 7308 (numbering as in ref. 2) of the DNA sequence of the PC gene.

The amplified fragment was submitted to DGGE on a 30–80% gradient of denaturing agents (100%-7M urea and 40% formamide in TAE buffer) at 160 Volts for 5 hours.

Polymorphism: As demonstrated in figure 1, we detected a two allele polymorphism. Direct sequencing of the PCR product obtained after asymmetric PCR showed the presence of a T (F1) or a C (F2) at nucleotide 7228 in codon corresponding to Asp 214 of the mature PC.

Frequency: We tested 76 unrelated individuals. The 7228 T (F1) allele has a frequency of 64% and the 7228 C (F2) allele of 36%. The observed heterozygosity is of 49%. The calculated PIC = 0.35.

Chromosomal Localization: PROC has been localized to 2q13-q21 (3–5) by in situ hybridization.

Mendelian Inheritance: Co-dominant segregation demonstrated in 6 families (33 individuals).

Clinical Relevance: Segregation studies in families with PC deficiency associated with thrombotic complications.

Acknowledgements: This work was supported by a research fellowship from La Fondation pour la Recherche médicale, and by a grant from l'Association Française contre les Myopathies. We wish to thank the doctors Lerman and Silverstein who kindly provided us the computer program.

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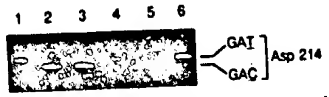

DGGE of amplified exon VIII of the PC gene: Lanes 1 and 6 are individuals homozygous for codon GAT corresponding to Asp 214. Lanes 2 and 3 are individuals homozygous for codon GAC. Heteroduplexes are seen in individuals heterozygous for each allele (Lanes 4 and 5).

A sequence polymorphism in the human peripherin/RDS gene

G.J.Farrar, P.Kenna, S.A.Jordan, R.Kumar-Singh and P.Humphries
Department of Genetics, Trinity College Dublin, Dublin 2, Ireland

Introduction: We report a silent polymorphism in the coding sequence of the human peripherin/RDS gene, which can be rapidly typed using allele specific oligonucleotides (ASOs).

Source/Description: Single strand conformation polymorphism electrophoresis and direct sequencing were used to identify sequence alterations in DNA fragments amplified from the human peripherin/RDS gene (1).

Polymorphism: We identified a C→T sequence polymorphism at position 558 in the coding sequence of the peripherin/RDS gene, which does not alter the amino acid sequence. To type the polymorphism, the region around the polymorphic site was amplified using the polymerase chain reaction (PCR), amplified products were Dot blotted onto nylon membranes and hybridised to ASOs.

Primer Sequences: Amplification primers: 5' TGCTATCC-TGTGTCTTCAAC 3' and 5' GTCTGTGTCCCGGTAGTACT 3' ASOs: A1: 5' TATCTGTGTCCTCTTCAACA 3' and A2: 5' TATCTGTGTTCTCTTCAACA 3'

Frequency: Studied in 166 chromosomes of unrelated European Caucasians. Heterozygosity = 0.49%
E1: 0.554
E2: 0.446

Chromosomal Localisation: The human peripherin/RDS gene has previously been assigned to chromosome 6p12 (1).

Other Comments: PCRs were performed according to reference (2). Mutations in the peripherin/RDS gene have been implicated in causing autosomal dominant retinitis pigmentosa, an inherited human retinopathy (3). Therefore this newly identified polymorphism within the peripherin/RDS gene should prove to be extremely useful for linkage studies with retinitis pigmentosa families.

References: 1) Travis,G.H. *et al.* (1991) *Genomics* **10**, 733–739. 2) Farrar,G.J. *et al.* (1990) *Am. J. Hum. Genet.* **47**, 941–945. 3) Farrar,G.J. *et al. Nature* (in press).

241594

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Human Molecular Genetics, Vol. 1, No. 9 769–771

Autosomal dominant Retinitis Pigmentosa: a novel mutation in the rhodopsin gene in the original 3q linked family

G.J.Farrar*, J.B.C.Findlay[1], R.Kumar-Singh, P.Kenna, M.M.Humphries, E.Sharpe and P.Humphries

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Received August 10, 1992; Revised and Accepted September 28, 1992

Retinitis Pigmentosa (RP) describes a heterogeneous group of retinopathies primarily involving photoreceptor degeneration (1). Linkage studies have enabled the localisation of disease causing genes. Two RP genes have been mapped to Xp (2). The first autosomal gene mapped to 3q, close to the gene encoding the photoreceptor cell specific pigment rhodopsin (1). Mutations within the rhodopsin gene have now been implicated in both autosomal dominant (adRP) and autosomal recessive (arRP) forms of RP (1, 3). More recently, an adRP gene has been localised to 6p, close to the gene encoding the photoreceptor protein peripherin/RDS (1). Mutations in this gene have been shown to co-segregate with the disease phenotype in a number of adRP families (4–6). Still further heterogeneity exists within adRP. It has been well established that there is an adRP locus at the pericentric region of chromosome 8 (7). There is also weak evidence for the involvement of a second adRP gene on 3q (8).

We have now identified a new rhodopsin mutation in the original family (TCDM1) used to localise the first adRP gene to 3q. Using heteroduplex analysis (9), we observed that heteroduplex DNA was present in only affected individuals in DNA fragments amplified from exon 3 of the rhodopsin gene. Direct genomic sequencing revealed that this was due to a T→G base change at codon 207, which results in the substitution of a methionine residue for an arginine residue. Using an allele specific oligonucleotide based assay, we established that the disease phenotype and the mutation were co-inherited (Figure 1), with a maximum Lod score of 17.7 at zero recombination (Table 1a). We tested 100 normal unrelated individuals for the presence of this mutation and found that it was absent in all these controls.

This finding is of particular interest in view of weak evidence suggesting the possible involvement of a second adRP locus on 3q (8). Using rhodopsin mutations in adRP families as highly polymorphic markers Inglehearn and colleagues observed 12% recombination ($\theta = 0.12$, $Z = 4.5$) between the rhodopsin gene and the marker (D3S47) on 3q. They suggested that the presence of some families in which no rhodopsin mutations had been found but which showed tight linkage between adRP and D3S47 was consistent with the hypothesis that there may be a second adRP locus on 3q. Our recent identification of a rhodopsin mutation in TCDM1 must lend some doubt to the suggestion of two adRP loci on 3q, which was based on families such as this one.

The clinical presentation of adRP in the TCDM1 family is of particular interest due to the uncharacteristically early onset of the disease when compared to other well documented adRP patients (10). Affected members exhibited onset of the disease within the first decade of life, diffuse funduscopic disturbances, and extinguished rod photoreceptor cell responses as assessed by ERGs. However ERG cone responses were retained until the third decade, although significantly reduced in amplitude and delayed in latency. Two colour dark adaptometry indicated a diffuse loss of rod and cone photoreceptor sensitivity with a greater involvement of rods and hence mirrored the electroretinographic findings.

The mutations observed within the rhodopsin gene and implicated in human inherited retinopathies have an extremely broad clinical spectrum. We can use previously constructed models of rhodopsin to speculate on the structure/function relationships of mutant rhodopsin molecules and hence possibly gain some insights into the underlying basis of the wide clinical spectrum observed in rhodopsin linked RP.

There is a considerable body of data from protein chemistry and site-directed mutagenesis which can be amalgamated to provide a crude representation of the 3-D structure of rhodopsin. Such approaches can never provide a detailed description of the conformation of the protein but they can augment our understanding of its structure and provide a model for investigating it's mechanisms of action. From such studies it is possible to speculate on the importance of the Met 207→Arg substitution. There are two feasible scenarios. The first is that the methionine side-chain is exposed to the hydrophobic environment of the bilayer or is the interface between two transmembrane segments. Substitution by an arginine would seriously perturb folding either by destabilising the protein with a potentially charged residue to the hydrophobic phase or by a bulky polar residue at a helix–helix surface. An alignment of all rhodopsin sequences in this region indicates that Met 207 can be substituted by other hydrophobic residues and by Tyrosine (Table 1b). At first sight then, one could suppose that the residue is not critical to the intersegment contacts and could therefore be exposed to lipid.

An alternative explanation is illustrated in Figure 2. This shows the position of the methionine side chain relative to the retinal models derived from theoretical considerations of hydrophobicity and conservation (Donnelly,D. and Blundell,T., unpublished) or from the modification of residue side-chains by hydrophillic and hydrophobic probes (11). From this model it can be seen that the Cα and Cβ atoms of Met 207 are of the order of 4 Angstroms

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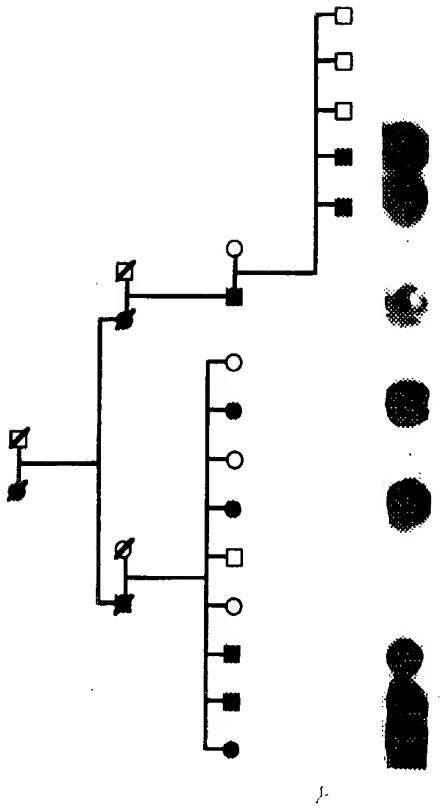

Figure 1. Amplified DNA from affected and unaffected individuals from TCDM1 was hybridised to the both the normal (5'TGTCATCTACATGTTCGTGG3') and mutant (5'TGTCATCTACAGGTTCGTGG3') allele specific oligonucleotides (ASOs) around codon 207. In this figure for convenience only a part of this large family is shown hybridising to the mutant ASO. Only affected members of the family hybridised to the mutant ASO (A).

Table 1. a. Two point Lod scores between the disease gene and the rhodopsin codon 207 mutation segregating in TCDM1. **b.** Alignment of residues 202–228 in 20 visual pigment sequences. This region of the protein is likely to contain the 5th transmembrane segment.

a.

θ	0.4	0.3	0.2	0.1	0.05	0.00
2pt-Lod scores	3.54	7.72	11.42	14.71	16.23	17.68

b.

SFVIY	M	FVVHFIIPLIVIFFCYGQLVF	BOVINE
SFVIY	M	FVVHFSIPLIVIFFCYGQLVF	OVINE
SFVIY	M	FVVHFTIPMIVIFFCYGQLVF	MOUSE
SFVIY	M	FVVHFTIPMIIIFFCYGQLVF	HUMAN
SFVIY	M	FVVHFMIPLAVIFFCYGNLVC	CHICKEN
SYVVY	M	FVVHFLVPFVIIFFCYGRLLC	LAMPREY
SYMIV	L	MVTCCIIPLAIIXLCYLQVWL	HUMAN-RED
SYMVV	L	MVTCCFFPLAIIILCYLQVWL	CHICKEN-RED
SYNIV	L	MITCCFIPLGIIILCYIAVWW	FISH-RED
SYMIV	L	MVTCCITPLSIIVLCYLQVWL	HUMAN-GREEN
SYMVT	L	LLTCCILPLSVIIICYIFVWN	FISH-GREEN1
SYXIT	L	MLTCCILPLSIIIICYIFVWS	FISH-GREEN2
SYTWF	L	FIFCFIVPLSLICFSYTQLLR	HUMAN-BLUE
SNILC	M	YIFAFMCPIVVIFFCYFNIVM	SQUID
SFILC	M	YFCGFMLPIIIIAFCYFNIVM	OCTOPUS
SYLIF	Y	SIFVYYIPLFLICYSYWFIIA	DROSOPHILA-RH1
SYLIF	Y	SIFVYYLPLFLICYSYWFIIA	CALLIPHORA-RH1
SYLIT	Y	SLFVYYTPLFLICYSYWFIIA	DROSOPHILA-RH2
LFVAC	I	FFFSFVCPTTMITYYYSQIVG	DROSOPHILA-RH3
LFVGT	I	FFFSFVCPTLMILYYYSQIVG	DROSOPHILA-RH4

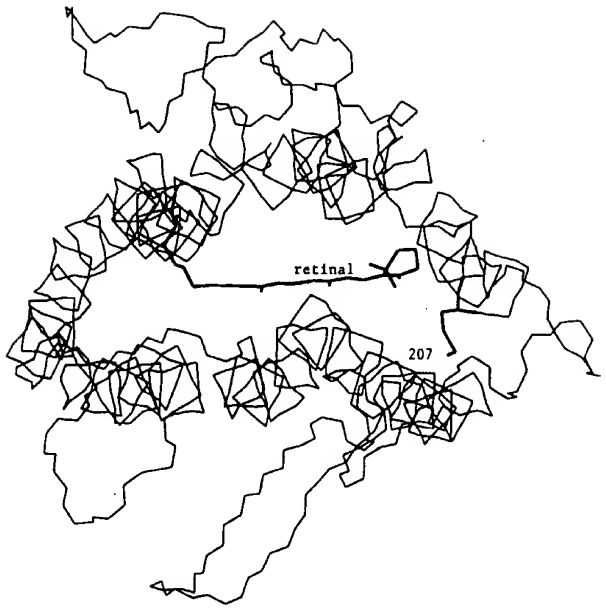

Figure 2. Transverse section through the model of rhodopsin to illustrate possible positions for the chromophore and the side-chain of Met 207. The Cα and Cβ positions of the later are fixed but the other atoms can rotate. The attachment point of the aldehydic group of 11-cis retinal is on helix 7 (top left), rotating clockwise through helices 6 to 1 (extreme left).

from the ring of retinal depending on the position of the chromophore. This does not necessarily imply that a significant interaction between the two occurs but it does suggest that there is not much scope to accommodate a bulky polar residue in this region without seriously perturbing the binding of the chromophore, leading in turn to an inactive receptor. It is perhaps relevant that the same position in the beta-adrenergic receptor is occupied by a serine residue, mutation of which affects the ability of the protein to interact with it's ligand (12). The previous residue is also a serine and again substitution by alanine has a marked effect, this time on the folding of the polypeptide. Clearly this whole region is critical to the structure and activity of the protein and it is important to determine experimentally the effect of the Met 207→Arg substitution in human rhodopsin.

The exact means by which mutant rhodopsins result in photoreceptor degeneration have still to be elucidated. To proceed from the level of observation of rhodopsin mutations in RP patients, to an understanding of the underlying basis of these retinopathies, it will be important to investigate the altered activities and the structure/function relationships of mutant proteins in detail. Previously developed models may serve to direct these studies. In turn, the observation of amino acid changes within the rhodopsin protein which have radical phenotypic effects in humans will provide valuable information for those updating models of the rhodopsin protein.

ACKNOWLEDGEMENTS

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concerted action programs of the Commission of the European Communities and the Health Research Board of Ireland. Clinical assesment of patients was carried out at the Research Department of the Royal Victoria Eye and Ear Hospital, Dublin.

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4830 — 9:30

PROGRESS IN GENETIC LINKAGE FOR RETINITIS PIGMENTOSA AND GENE DELIVERY TO OCULAR TISSUES

((G.J. Farrar[1], P.F. Kenna[1], F. Manserghi[1], A.S.W. Erven[1], M.M. Humphries[1], S. Kennedy[2], P. Sieving[3], R. Bush[3], K. Gulya[4] and P. Humphries[1])). The Wellcome Ocular Genetics Unit, Trinity College Dublin, Ireland[1]; Research Dept., Eye and Ear Hospital, Dublin, Ireland[2], Kellogg Eye Center, University of Michigan, Ann Arbor, USA[3]; Dept. of Zoology and Cell Biology, Attila Jozsef University, Szeged, Hungary[4]

Purpose To localise disease genes for inherited retinopathies and to investigate viral delivery of genes to ocular tissues. **Results** a/ Linkage studies: In one adRP pedigree with sensorineural deafness we excluded areas of the genome to which autosomal dominant RP (adRP) genes have previously been mapped, hence providing evidence for the presence of an eighth adRP gene. To date, over 200 microsatellite markers have been analyzed in this family. We have studied two pedigrees with Best's macular dystrophy. In one we refined linkage around 11q13, the region to which the disease gene maps. In the second we excluded the disease gene from 11q13 indicating the presence of genetic heterogeneity. In addition, the location for an adRP gene on 7q has been refined using 21 microsatellites from a 15cM region. b/ Gene Delivery: The characterisation of a number of genes for inherited retinopathies has stimulated us to investigate methods of delivering genetic information to ocular tissues, the goal being to proceed towards gene therapies for these disorders. Initial studies have involved the use of adenovirus carrying the Lac Z reporter gene driven by the CMV promoter. Both vitreous and sub-retinal injection procedures have been undertaken on mice of various ages. The range of tissues infected and the longevity of expression of transferred genes was monitored for 2 months post injection. Progress in these areas will be discussed. (Supported by the Wellcome Trust, the US National, British and Irish RP Societies, the EU Science Program and the Gund and Ulverscroft Foundations).

None

4831 — 9:45

A YAC CONTIG ENCOMPASSING THE USHIC LOCUS ON THE SHORT ARM OF CHROMOSOME 11.

((R. Ayyagari[1], R.J.H. Smith[2], M. Polymeropolous[1], S. Daiger[1], M.Z. Pelias[2], L. Wozencraft[1], M. Kaiser-Kupfer[1], A. Nestorowicz[3], A. Permutt[3], Y. Lee[1], J.F. Hejtmancik[1].)) National Eye Institute, NIH[1]; Department of Otolaryngology, University of Iowa[2]; National Center for Human Genome Research, NIH[3]; The University of Texas Health Science Center at Houston[4]; Department of Biometry and Genetics, Louisiana State University Medical Center[5]; Department of Internal Medicine, University of Washington in St.Louis[6].

Purpose. To construct a YAC contig covering the Usher Syndrome type 1C locus on the p arm of chromosome 11. **Methods.** Linkage analysis places USH1C gene to a 5.5 cM region between D11S861 and D11S899 on the p arm of chromosome 11. YAC clones in this region were identified using 2 new STS markers developed and the microsatellite markers in this region. **Results.** A YAC contig has been constructed covering the critical region of USH1C and spanning over 7 loci: D11S926, D11S1307, D11S861, D11S419, D11S902, D11S1397 and D11S921.STS were derived from the insert sequences of the YAC clones to identify the overlapping YACs. Location of the STSs was confirmed using the 11 somatic cell hybrid panel. The order of the microsatellite markers on the YAC contig confirms the order on the genetic map. Seven additional YACs were identified with D11S1310 and D11S899 to extend the contig further centromeric to the existing region. Additional YACs were identified from CEPH libraries using an STS derived from the end sequence of YAC present at the end of the contig. Analysis and characterization of these YACs is under progress to further strengthen the contig. **Conclusions:** A YAC contig covering the USH1C on the p arm of chromosome 11 has been constructed using YACs identified. This should facilitate identification and cloning of the USH1C gene.

4832 — 10:00

JUVENILE RETINOSCHISIS (RS): REFINED X-CHROMOSOME MAPPING.

((H.S. Pawar, E.L. Bingham, K.L.Lunetta, J.E. Richards, M. Boehnke & P.A. Sieving)) Ophthalmology, Epidemiology, Biostatistics: University of Michigan.

PURPOSE: Juvenile X-linked Retinoschisis (RS) typically causes central and peripheral vision loss beginning early in life. Intraretinal cysts form around the fovea, and the peripheral retina splits through the nerve fiber layer. The RS gene has been localized to the Xp22.1-p22.2 region on the X-chromosome. We are working to isolate the RS gene using positional cloning and have narrowed the genetic interval around the RS locus. This will be helpful in constructing a refined genetic and physical map of the region.
METHODS: Nine microsatellite repeat markers were used for linkage analysis of our 43 RS families. Standard PCR amplification was used, and analysis was carried out using the method of lod scores.
RESULTS: We previously localized RS between (DXS43,DXS987,DXS207) on the telomeric side and DXS41 on the centromeric side. We have now used several new microsatellite repeat markers to study RS linkage in this genetic interval between DXS43-DXS41. In eight large RS families with more than 120 meioses, lod scores in excess of 20 were obtained for DXS418, DXS207, DXS987 and DXS999. Recombinations were found between RS and (DXS207, DXS987 & DXS43) on the telomeric side. Additional markers (DXS1226, DXS274, DXS1052, DXS443 and AFM291wf5) were also used for analysis of key recombinant individuals. The closest centromeric markers were (AFM291wf5, DXS443).
CONCLUSIONS: This study narrowed the RS interval by about 5.7 cM on the centromeric side, from DXS274 to (AFM291wf5, DXS443). The telomeric boundary remained at (DXS43,DXS987,DXS207). Haplotype analysis suggested the order (DXS43,DXS987,DXS207) - RS - (AFM291wf5, DXS443) - DXS1052 - (DXS1226, DXS41). The genetic distance between DXS43 and AFM291wf5 is about 3.8cM, and this interval should contain the RS gene. We have YAC clones that cover the RS region and are searching for expressed sequences to evaluate as candidate genes. One 1.2 mb YAC clone showed the presence of DXS999, AFM291wf5 and DXS443.
Support: NIH EY10259 (PAS); RP Foundation, Baltimore, MD. (none)

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Gene transfer into neurones: from basic applications to gene therapy

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ASSESSMENT OF LIPOSOMAL TRANSFECTION OF OCULAR TISSUES *IN VIVO*

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Summary

A possible route to the treatment of inherited retinal degenerations such as retinitis pigmentosa (RP) is the application of somatic gene therapy by the transfer and expression of corrective functional genes in ocular tissue. Cationic liposomes are established vehicles for the delivery and expression of exogenous genes in mammalian cells both *in vitro* and *in vivo*[1-4]. We report here a preliminary assessment of liposome-mediated transfer of a plasmid carrying the reporter gene *lacZ* (encoding the enzyme β-galactosidase) into tissues of the adult rabbit eye.

Materials and Methods

Adult New Zealand White rabbits (weight, 2.5-4.0 kg) were anaesthetized with fentanyl citrate and diazepam. Two routes of injection were used. (1) Following conjunctival peritomy of the right eye, a sclerotomy was performed 10mm posterior to the limbus midway between the superior and lateral rectus muscles. The choroid was exposed and incised, and a Hamilton syringe needle introduced into the subretinal space. (2) Intraocular access was gained through a pars plana sclerotomy. A retinotomy was fashioned, through which the preparation was injected using a lacrimal cannula, and a retinal bleb raised.

The DNA used for transfection was the plasmid pCH110, which carries a reporter gene encoding *E.coli* β-galactosidase driven by an SV40 early promoter. Rabbit right eyes were injected with mixtures containing 1:1 ratios (w/w) of plasmid DNA and the transfection reagent DOTAP[5] or Lipofectin[6] (n_{test}=9), or controls of liposome only, pCH110 only, saline only, or DOTAP and a non-reporter plasmid ($n_{controls}$=5) were injected. Additionally, in 3 cases, blue latex microspheres (average diameter 0.24 μm) were added to the transfection mixture to mark the route of injection. The left eye served as untreated control for each animal.

Forty-eight to 120 hours post-injection the rabbits were killed and the eyes enucleated, fixed in 4% paraformaldehyde/ 0.1M sodium phosphate buffer pH 7.4 and incubated in 30% sucrose/0.1M phosphate buffer overnight and frozen in isopentane. Cryostat sections (10 μm thick) were treated histochemically with the substrate X-gal to detect β-galactosidase activity[7]. Eyes injected with mixtures carrying coloured latex microspheres were also examined macroscopically following removal of the anterior segment.

Results

Both routes of injection produced partial retinal detachment with bleb formation enclosing the liposomal mixture. The track of injection in the vitreous was visualized by the coloured microspheres.

At the light microscopic level, specific intracellular blue reaction product, indicative of functional β-galactosidase, was observed in sections of all test injected eyes (pCH110 + liposomes). Numerous positive cells were seen in the outer part of the choroid, including the region of the suprachoroidea and lamina fusca. In certain cases, strong specific reactivity was also seen in the ciliary body. The greatest focus of intensity was found in the layer corresponding to the outer epithelial layer of the ciliary processes (which would be pigmented in non-albino animals), and particularly in the basal half of these processes.

These patterns of positive reaction were not observed in sections of any of the injected or non-injected control eyes.

Discussion

We wished to investigate the possibility that exogenous genes may be expressed in ocular tissues *in vivo*, as a first step towards corrective strategies for the treatment of inherited eye disease. Here we report an assessment of liposome-mediated transfer of a plasmid carrying the reporter gene *lacZ*, driven by the SV40 early viral promoter, to provide evidence of transfer and expression of the active, encoded enzyme β-galactosidase in cells of rabbit ocular tissues. The primary target for such expression would be the photoreceptor cells, since photoreceptor degeneration has been associated with defects in genes expressed particularly in these cells (for review see e.g. Humphries *et al.*[8]).

Under the conditions of subretinal injection and preparations of plasmid DNA-liposome complexes used in this study, specific expression of the reporter gene was observed in rabbit ocular tissue. Expressing cells were seen primarily in the choroid and in the outer epithelium of the ciliary body. These regions did not show similar expression in any of the control eyes. Electron microscopy to identify the types of cell showing uptake and expression is now in progress. In the choroid, the most probable candidate types are amelanic melanocytes and infiltrating macrophages. It is possible that that the SV40 promoter is functional in these cells, and in the outer epithelial cells of the ciliary processes, in preference to other cell types.

To improve targeting of delivery and expression of reporter and photoreceptor genes to the neuroretina, we are currently assessing modifications of the promoter and liposomal constructs.

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PROGRESSIVE RETINAL ATROPHY: A MODEL FOR RETINITIS PIGMENTOSA IN COMPANION ANIMALS

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Abstract
The generalised progressive retinal atrophies (PRA's) are a heterogeneous group of inherited retinopathies in dogs and cats. They show marked similarities to the retinitis pigmentosas (RP) in man, as well as to a number of inherited retinal degenerations of laboratory rodents. Of the various recessively inherited forms of PRA in the dog, that in the Irish setter is due to a nonsense mutation in the gene encoding cGMP-PDE-β subunit. Mutations at this locus are also known to cause a proportion of human RP cases. We are interested in applying gene therapy tothese diseases.

Retinitis Pigmentosa and Progressive Retinal Atrophy
In humans the RP's are an important cause of blindness, affecting about 1 in 4000 of the population. They are a group of inherited retinal degenerations or dysplasias with onset usually in childhood or early adulthood. Between 20% and 50% of RP cases show recessive inheritance, with the remainder showing either dominant or X-linked modes of inheritance[1]. A number of different loci, including those encoding the photopigment rhodopsin and a photoreceptor cell outer segment specific structural glycoprotein known as rds-peripherin, as well as at least 2 others of unknown function have been implicated in autosomal dominant RP[234]. In addition at least 2 loci have been linked to X-linked RP[5]. Investigation of autosomal recessive RP are less well advanced, but the loci encoding rhodopsin[6] and rod specific cGMP-phosphodiesterase (β subunit)[7]have been linked to this disease in a small proportion of cases investigated. Mutations in the genes for peripherin[8] and cGMP-PDE-β[9] have also been shown to cause recessively inherited retinal degenerations in laboratory mice.

The generalised PRAs in dogs are a group of primary photoreceptor diseases which are distinguishable genetically (by complimentarity) in different diseases[10]. The forms investigated so far have all been recessively inherited. In contrast, the Abyssinian cat shows both dominant and recessive forms of PRA. Clinically the signs of each type of generalised PRA are similar, although the age of onset differs. Histopathological changes also differ in detail between the various forms, but in each case are progressive, resulting in eventual degeneration of all retinal layers. Affected animals, like humans with RP, show initial night blindness, followed by progressive loss of day vision, eventually resulting in total blindness. The companion animal PRA's are a particularly useful model for human autosomal recessive RP(ARRP) for at least 3 reasons:
i) Pedigree breeds which suffer from these conditions are often extensively inbred: there are large pedigrees available, and the phenotype can be followed through many generations, making the acquisition of cases and linkage analysis rather easier than it often is in the human case.
ii) Many (at least 5) non complementing loci have been linked to the disease. An understanding of each of these conditions may give an insight into some forms of human ARRP.
iii) Research colonies of several breeds of dog and cat suffering from PRA are available. This resource allows close study of the development and biochemistry of the defects.

Aetiology of Progressive Retinal Atrophy
We have used a candidate genes approach to examine the aetiology of PRA. We have recently cloned both the canine rod-specific opsin gene[11] and the canine cGMP-PDE-β gene. We have begun screening these two genes for mutations in the various canine PRA models. As yet we have not detected any polymorphisms showing linkage to PRA in the opsin gene, but we have recently reported a nonsense mutation in position 2420 of the cGMP-PDE-β gene of Irish setters which segregates with an early onset form of PRA known as rod-cone dysplasia type 1 (rcd-1)[12]. This G > A transition creates a tryptophan to amber mutatation at codon 807 which causes premature termination of the protein 49 amino acids upstream of its normal terminus, and would be expected to abolish the ability of this subunit to bind to the rod outer segment membrane. The same mutation has also recently been reported in an American colony of Irish setters suffering from rcd-1[13]. However, it is not found in Tibetan terriers, long haired dachshunds or miniature poodles with later onset forms of PRA.

Gene therapy and the progressive retinopathies
When considering gene therapy in relation to the inherited retinopathies there are several difficulties:
i. Currently, gene replacement strategies are only useful in the case of recessive disease. Of the currently understood human RP's only those with mutations in the cGMP-PDE-β locus and a small proportion of those with mutations in the opsin locus obey these criteria.
ii. The affected cells in these diseases are the photoreceptor cells, and in most cases specifically the rod cells. It is likely that effective repair will require gene expression in a largeproportion of these cells. We do not currently have good non-transgenic methods to deliver genes to these cells. The mouse is not an ideal model for the human in such studies because of the differences in rod and cone frequency, in state of retinal development at birth and in sheer size in the 2 species. In all of these respects, companion animals are arguably a half-way house between mouse and humans.

We are interested in developing models for targetting genes to the murine and subsequently the canine rod cell with the long term aim of applying these to RP in humans.

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Minireview

Recent developments in certain X-linked genetic eye disorders

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(Received 5 January 1993)

Key words: Recombinant DNA; Restriction fragment length polymorphism; Genetic map; Eye disorder; X-linked

Contents

Summary

Over the past few years, genetic diseases of the ocular system have become very active and fast-growing research areas in the vision field. The rapid development of the recombinant DNA techniques together with somatic cell genetics, during the last two decades has fueled this progress. As a result, many genetic disease genes have been localized in the human chromosome and several of them have been isolated and characterized. These and other studies have profoundly enriched our basic understanding of genetic eye disorders. Although gene replacement therapy, prenatal diagnosis and carrier detection have not been extensively tried for genetic eye diseases, such attempts will now be feasible. Molecular analyses made it clear that there are many challenging problems that need attention. This report highlights some of these initial developments, particularly on the X-linked major genetic eye diseases. In order to help the beginners and general audience, a brief description of the clinical pathology and the molecular probes used to locate the genetic defects of certain disorders are presented. Disorders are arranged according to their linkage from telomere to telomere on the chromosome to give a coherent structure. It is hoped that this information is useful and of general interest for the beginners, established investigators and ophthalmologists.

I. Introduction

Blindness or visual impairment is one of the devastating problems which people fear most. Each has always been a major public health concern. It is estimated that approximately 30 million people in the world are blind [1] and a similar number are reported to be visually disabled. Serious eye problems can interfere with education, physical development and destroy careers. In children blind before the age of 6, 50% are believed to have an inherited disease [2]. In these cases, especially where clinical data may be hard to

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obtain, and in other inherited diseases where data are inconclusive, a combined knowledge of clinical and basic genetic research may permit the physician to diagnose the disease more accurately. This is because DNA testing does not require clinical information. Additionally, the availability of a DNA test may reduce the need for other more extensive and expensive testing. This is particularly true in the case of mild forms of the diseases because it is hard to differentiate a mild form of a common genetic disease from a distinct genetic disorder. In fact, the lack of genetic knowledge in several instances is unfortunate for the many individuals who have inherited vision loss. Therefore, genetic research in inherited eye diseases is such a worthy one that it will help in obtaining the details of the ocular pathogenesis and possibly to develop a better therapy or prevention.

In recent years the application of molecular genetic techniques to the study of genetic diseases has provided a better understanding of human genetic disorders at the molecular level. During the course of the last 20 years, a whole spectrum of disease genes have been identified, cloned and characterized. Diseases such as cystic fibrosis, Duchenne muscular dystrophy and neurofibromatosis are now amenable for diagnosis at the DNA level [3]. Similar advances have also become possible in the eye field. During the 1980s remarkable progress was made in localizing, isolating and characterizing some of the genes responsible for certain heritable eye diseases. Recently, the amplification of the target sequences (PCR) enabled investigators for the first time to localize the point mutations in the rhodopsin gene of some autosomal dominant retinitis pigmentosa. Similarly, genetic linkage analyses helped to map the Usher syndrome (type II) to the long arm of chromosome 1. Although these and other studies have profoundly enriched our basic understanding of the genetic eye disorders, the number of eye diseases that can be definitely diagnosed by DNA analysis is still quite limited. In this article, I have attempted to highlight some of these developments, particularly on the X-linked major genetic eye diseases, because of their general interest for researchers and ophthalmologists. Disorders which are either extensively studied or newly assigned or for which candidate genes are available have been selected for the discussion in this article (Table I) and several other disorders which are known to affect the eye are summarized in Table II for completeness. No attempt has been made to cover the entire field exhaustively or any particular disease in detail. In addition, several systemic diseases which are also known to affect the ocular system, autosomal and some of the X-linked eye disorders are not included in this essay, since a descriptive compilation of these has recently appeared [4,5].

It is appropriate to begin with a brief outline of different parts of the eye and its function. The visual system is a highly organized and complex system. The optical element of the eye, the lens and the cornea (Fig. 1) focus image on the retina which converts the incoming light to electrical signals which are further processed by a portion of the brain. Most blindness and visual disability is caused by disorders of the retina and choroid. Therefore, a major portion of the discussion is devoted to those disorders which primarily affect the retina and the choroid.

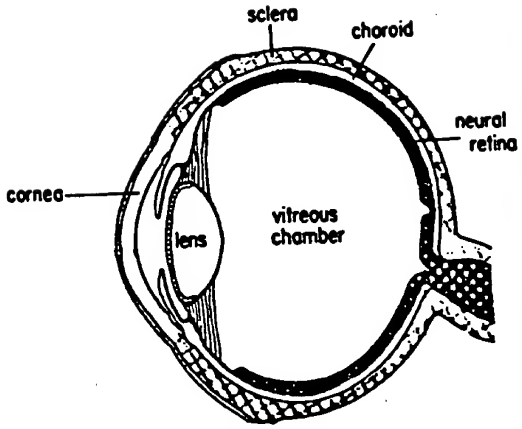

Fig. 1. Diagram of a horizontal section of the human eye showing the organization of the cornea, lens and the retina. The lens and the cornea focus image on the retina which converts the incoming light to electrical signals. The retina is the innermost of the three coats that form the wall of the eyeball. The vitreous, normally a gelatin-like substance which fills the larger chamber in the back of the eye protects the globe from collapsing and allows the unhindered transmission of light to retina.

II. Ocular albinism

This is a heterogeneous group of heritable disorders of the melanin pigment system of the eye. Macromelanosomes are characteristic features of the X-linked recessive form which are not seen in the autosomal recessive form. In an X-linked trait there are two types of ocular albinism, designated OA1 and OA2, which are also called Nettleship-Falls type [6,7] and Forsius-Eriksson type, respectively [8–10]. The affected individuals show decreased visual acuity, hypopigmentation of retina and hypoplasia of the fovea and have normally pigmented skin. Carriers may also show pigmentation in the periphery of the fundus and the presence of translucent irises. Molecular genetic studies using multiplex polymerase chain reaction amplification mapped the locus for OA2 to the sub-bands Xp21.3-21.2 between the marker DXS67 and Duchenne muscular dystrophy [11]. However, in the case of OA1, familial cosegregation was observed between OA1 and X-linked ichthyosis which is caused by the deficiency of

steroid sulfatase enzyme. By using cloned DNA probes the locus responsible for OA1 disorder has been mapped to Xp22.2-p22.3 region and has the following gene map: DXF30S1-STS-DXS237-DXS143-DXS16. The position of OA1 is likely to be in between DXS 237 and DXS143 [12]. Because of the extreme clinical variability of this disease, accurate genetic counseling for the carrier is difficult. However, these studies should prove invaluable for the ultimate goal of identification and characterization of the genes responsible for ocular albinism.

III. Retinoschisis

Retinoschisis belongs to the still poorly defined group of vitreoretinal dystrophies. Congenital hereditary retinoschisis is inherited through an X-linked recessive mode and hence occurs exclusively in male patients. The affected individuals invariably show foveal retinoschisis, vitreous degeneration, abnormalities of the retinal vasculature [13] and a schisis of both peripheral and central retina. This is a progressive disease with slow deterioration that occurs throughout life, and with no known cure. Abnormalities of the retina can be detected in young patients at 3 months of age. Carriers of X-linked retinoschisis cannot be detected by clinical means. On the basis of histopathological studies and the abnormality in the electroretinogram [14–16] it was suggested that Muller cells may play a critical role in the pathology of this disorder.

Although the biochemical defects underlying this disease are not understood, linkage analyses performed using large numbers of families with several affected males [17,18] and highly polymorphic markers strongly suggest that this disease is linked to Xp22.2-p22.1 loci with the following gene map: ter-DXS16-(DXS207, DXS43)-RS-DXS274-(DXS41, DXS92)-cen. [17,18]. Many studies indicate a homogeneous etiology of retinoschisis, but it is not clear at present what contributes to the marked variation in its phenotypic expression. It is possible that different mutations or deletions in a single gene may contribute to the different clinical features.

IV. Cataract

A cataract is an opacity of the normally clear lens of the eye. In humans, opacification of the eye lens is the leading cause of visual impairment and blindness. Hereditary cataracts in the majority of cases are transmitted as autosomal dominant traits [19] and can be clinically heterogeneous even among family members. An X-linked congenital cataract [20] is a rare disorder and exhibits central cataract, microphthalmia or microcornea and dental abnormalities [21,22]. The congenital cataract associated with dental anomalies is also known as the Nance-Horan syndrome [19].

An extensive effort has been made to localize the defective regions responsible for this disorder. While there have been no chromosomal deletions or rearrangements detected, mapping techniques using restriction fragment length polymorphism (RFLP) [19,23,24] have shown that the disease locus resides at Xp21.1-p22.3. Similarly, cataract congenital total (CCT) has been assigned to the short arm of X-chromosome [25]. Further linkage analysis with other closely linked markers and the isolation and characterization of the candidate gene responsible for this disease may provide an explanation for the clinical variability of this disease.

V. Retinitis pigmentosa

Retinitis pigmentosa comprises a group of degenerative and progressive disorders of the photoreceptor cells of the retina which affects between 50000 to 100000 people in the United States. Although there is a great difference in the expression of this disease between different types of retinitis pigmentosa, the disorder is characterized initially by night blindness and progresses with color vision anomalies, retinal pigmentary atrophy, loss of mid-peripheral visual field and eventual loss of central and far peripheral vision. This ultimately leads to total blindness. Retinitis pigmentosa is phenotypically heterogeneous and can be inherited as an autosomal dominant, an autosomal recessive and an X-linked trait [26–29] with genetic heterogeneity existing within each hereditary pattern. In an X-linked disorder, only males in the pedigree are severely affected and there is no history of male to male transmission. Carriers of X-linked recessive RP show no evidence of disease.

In spite of an extensive effort, no candidate gene defect has been described for X-linked retinitis pigmentosa. However, linkage analysis of affected families suggests the presence of at least two [26,27] and possibly three disease loci. One of the loci (RP2) is closely linked to DXS7 and there are no reports of chromosomal translocation or deletions in RP2 disorder, but a possibility of centromere effect has been reported [30]. The second (RP3) maps near to the locus ornithine carbamoyl transferase [27]. This RP3 locus is supported by the fact that affected males show multiple phenotypes and deletions in the Xp21 region [26,31]. Interestingly, a family with RP3 type disorder has been found to be associated with ciliary abnormalities [32] which is consistent with its multiple phenotypic expression. The third putative locus is found distal to ornithine carbamoyl transferase [33] possibly at map position 76 cM, midway between DXS28 and DXS164 markers. The third locus is very closely linked to RP2

and has the following regional gene map: Xcen-OTC-RP3-DXS140 [26]. It is not clear at present whether the multiple loci are postulated because of a lack of highly informative markers or that these studies simply suggest the genetic heterogeneity and complexity of this disorder. This certainly is a challenging avenue for the future. Several loci are possibly contributing together to create a risk in a particular individual. Such multiple gene disorders are poorly understood. For a routine diagnosis however, more closely linked probes with defined chromosomal regions would be very helpful. From the practical standpoint, the availability of cloned materials or the sequence information of the gene is essential to provide genetic analysis of the disorder. For this purpose, the recently identified [34,35] hypervariable DNA marker M27 beta (DXS255) and microsatellite probes (DXS426) or the positional cloning technique may accelerate the cloning the proximal locus for the X-linked disorder. It will be gratifying in the future to learn how three different modes of inheritance produce the same clinical phenotype. This is because any mechanisms involving genetic abnormalities should be able to explain the tissue specificity of the disease and its delayed onset. In other words, all the genes involved should be retina specific. Alternatively, retinitis pigmentosa may represent an example of a general metabolic defect causing disease as has been suggested recently to explain other ocular disorders (see below).

VI. Norrie disease

Norrie disease is a congenital retinal dysplasia, clinically similar to retinopathy of prematurity. It is a rare genetic eye disease which can lead to congenital blindness, first recognized by Norrie [36] and characterized by a bilateral, retrolental ocular mass due to retinal dysplasia. Among other abnormalities several affected individuals show mental retardation, hearing loss, cataract, pseudoglioma and atrophy of iris [37–42]. This disease is inherited through an X-linked recessive mode and is often associated with several neurologic disorders of unknown pathogenesis. It affects only males and is transmitted through unaffected females. All affected males exhibit variable expressivity. DNA linkage analyses have mapped the Norrie disease gene to Xp11.3-11.2 and linked it to the marker DXS7 [37,39,43,44]. Interestingly, a submicroscopic deletion [38,42,45] involving the DXS7 locus and a familial pericentric inversion of the X-chromosome at bands 11.4 and q22 was observed in some patients having Norrie disease and other symptoms including mental retardation. Although a deletion of the monoamine oxidase gene has been noticed in some affected individuals [41,46–48], this is not found to be the case in a large family study having classic Norrie disease. Hence, it is unlikely that monoamine oxidase is involved in the pathogenesis of this disorder, although some secondary effect of metabolites cannot be ruled out at present. Additionally, as suggested by others [45,49,50], several different defective loci may contribute differently or in combination to the observed complex phenotypic expression of Norrie disease and some factors other than the gene defects themselves may be responsible for the severity of the disorder. A candidate gene which has been isolated [51,52] by positional cloning indicates homology between Norrie gene product and mucins [53]. This gene is found to contain microdeletions and point mutations in affected patients. Furthermore, the gene is found to be expressed in several tissues except in liver. Since an increased synthesis of mucins and alterations in the carbohydrates in these glycoproteins are known to play a critical role in the function and proliferation of tumor cells [54], it remains to be seen how Norrie gene product might be involved in clinical pathology of Norrie disease.

VII. Congenital stationary night blindness (CSNB)

This is a group of hereditary disorders of the retina in which the fundus appears normal by ophthalmoscopic examination, but the affected patients exhibit non-progressive night blindness, myopia and reduced visual acuity from birth. There are two types of congenital stationary night blindness. The first one is called complete type and is characterized by the night blindness, myopia and absence of rod function. The second is known as incomplete type and shows some functioning rods, reduced night vision and absence of myopia. This disorder can be inherited by autosomal dominant, autosomal recessive and X-linked recessive mode [55–58], and clinically there are great differences among each type. Both complete and incomplete types have also been found within the same X-linked pedigree. Carriers in X-linked disorder are not readily detectable by clinical means. By using eight multigeneration families and 17 polymorphic X-chromosome markers, the locus responsible for this disorder has been assigned to the region Xp11.3 [59–62]. Furthermore, using five point analysis the following order for the complete type: DXS7-CSNB1-TIMP-DXS255-DXS14 and for the incomplete type: DXS7-CSNB1-TIMP-DXS255-DXS14 and CSNB1-DXS7-TIMP-DXS255-DXS14 have been reported [62]. In a family which cannot be identified as either complete or incomplete type [63] multipoint analysis suggests the following map order: DMD-MAOA-CSNB-DXS426/TIMP. In any case, it is an interesting region of the X-chromosome, because two other genetic disease, namely retinitis pigmentosa and Norrie disease have been previously mapped to the same region. Although further experiments are needed to explore the effects and reasons for clustering of the

defects responsible for these three diseases, it appears likely that this region of the X-chromosome contains genes determining several different functions of the retina. Since myopia and nightblindness are co-inherited it is interesting to determine whether these are due to the same gene defect or due to tightly linked genes. Additionally, since an X-linked myopia without nightblindness and a separate inheritance of these two disorders in relatives [64] are known, it can be speculated that the disease phenotype may be caused by two different loci. Alternatively, it is possible that a single locus causes the night blindness, but the myopia is due to its secondary effect. It is also not clear at present whether the observed variation in clinical expression of X-linked disorder represents yet another example of genetic heterogeneity.

VIII. Choroideremia

Choroideremia (eremia = absence) is a rare heritable, bilateral, and progressive X-linked disease [65,66] causing central blindness in affected males during early adulthood. The affected individuals show progressive dystrophy of the choroid, retinal pigment epithelium and outer retina. Carriers regularly show an abnormal phenotype. The disease locus has been mapped to the Xq21 band [67–70]. With the use of positional cloning, which has recently become the classic approach to isolate human disease genes, a candidate cDNA clone spanning the same chromosomal region has been isolated [71]. DNA sequence analysis and characterization of this clone reveals that this gene is expressed in retina, choroid and retinal pigment epithelium. Its expression, however, is not restricted to the eye being found also in HeLa cells and lymphoblasts, although the defect is more specific for the eye. In addition, its transcript is either found to be absent or structurally altered in patients with choroideremia, suggesting the possibility that it may indeed represent the gene underlying the choroideremia. Moreover, point mutations that introduces termination into the open reading frame have been detected in patients with choroideremia [72]. A similar but not identical candidate cDNA for choroideremia has been reported recently [73]. Expression studies using this clone revealed no alteration in the genomic structure of this gene in the affected individual unlike those reported by others [71]. However, mRNA levels are found to be markedly reduced or absent in many patients. It is not clear at present which of these two cDNAs is an appropriate candidate gene for choroideremia. Interestingly, the protein encoded by the gene isolated by Cremers et al. exhibits significant (76%) similarity in its amino terminal region to the recently described p25A-GDI [74], and resembles the component A of rat Rab geranylgeranyl (GG) transferase [75,76]. One of these components is found to be missing in choroideremia [76]. Although the biological significance of this similarity awaits further experiments, availability of a cloned gene and elucidation of its function should help greatly in prenatal diagnosis and in understanding the molecular mechanisms responsible for the degenerative process of choroideremia. As suggested by Cremers et al. [71] choroideremia may be due to a widespread metabolic defect as in the case of gyrate atrophy and Refsum's disease.

IX. Fabry disease

Fabry disease was originally described by Fabry and Anderson and is also called Fabry-Anderson disease. It is an X-linked recessive, sphingolipid storage disease caused by deficiency in the activity of lysosomal hydrolase α-galactosidase A [77,78]. The affected males have no detectable α-galactosidase A activity and show a variety of systemic signs along with corneal opacities and cataract [79–80]. Isolation of cDNA and a genomic gene encoding α-galactosidase A (localized to the chromosomal region Xq21.33-q22) not only provided information regarding the structure and function of the enzyme, but also helped to identify six different germinal rearrangements [81] and point mutations [82–84] in the α-galactosidase gene in the affected individuals. However, development of a disease model in transgenic animals by using a rearranged or mutated gene or the introduction of an unmutated copy of the gene into a mutant cell line in vitro to correct the underlying biochemical and physiological defects have not yet been reported.

X. Lowe oculocerebrorenal syndrome

This disorder was originally described by Lowe [85] and is inherited as an X-linked recessive trait. The affected males exhibit congenital cataract, glaucoma, corneal opacities and progressive renal tubular dysfunction [86–88]. In addition, growth retardation and severe mental retardation have also been noted. Female carriers are known to have lens opacities but exhibit normal neurological and renal functions. Although the possibilities of several biochemical abnormalities have been reported [89] the basic biochemical defects underlying this disorder are unknown. Using several multi-generational families DNA based linkage analyses revealed that the gene(s) responsible for this disorder is closely linked to Xq24-q26 [86–88,90]. Recent isolation of yeast artificial chromosome clones of the human X-chromosome has helped to identify a candidate cDNA for this disorder [91,92]. There is no detectable genomic rearrangement and the affected individuals do not have an abnormal transcript of this gene. Based on the similarity of the encoded protein, it

has been suggested that Lowe syndrome is likely to be due to an inborn errors of inositol phosphate metabolism. Availability of these clones together with the application of polymerase chain reaction technology and the rapid identification of sequence variation should speed up the molecular characterization of this disease at the gene level.

XI. Color blindness

Human color vision is determined by the presence of a family of photopigments – the light absorbing molecules. The inheritance of color vision abnormalities has long been noticed, and several forms are now recognized. Among these, the red/green abnormalities are found to be the most common form. The loci responsible for this defect are mapped to the distal part of the Xq arm and are closely linked both to each other and to glucose-6-phosphate dehydrogenase [93–97]. To understand this disorder at the molecular level the cDNAs and genomic genes encoding blue, green and red photopigments have been isolated [94,95]. Surprisingly, in color normal males one copy of red cone and 3–5 copies of the green cone opsin genes are found to be present. Further characterization of these clones, together with the comparison of their structure and organization with that of the affected individuals, shows that red-green color blindness is due to genetic alterations (either gene conversion or unequal homologous recombination) of the genes encoding red and green photopigments. Interestingly, genetic variation in color matching with normal color vision is reported to be due to a common single amino acid polymorphism in red opsin gene [98]. Further studies involving 64 color-defective males have shown that Protan color-vision defects are associated with 5′ red-green hybrid genes and Deutan defects are due to green-pigment gene deletion [99]. In addition, defective color vision is reported to be due to missense mutation in the green visual pigment gene [100]. The availability of the well characterized color vision genes should help in understanding the mechanisms involved in the early photochemical events of human color perception. Additionally, this will also enable the investigator to understand blue cone monochromasy [97] which is an infrequent X-linked disorder [101,102] mediated entirely by the blue sensitive cone [103]. The provisional assignment of the locus for this disorder is found to be in the vicinity of Xq28, very close to the red and green cone opsin genes. To account for the blue cone monochromasy and red and green cone functions two possibilities have been suggested [93,97]: (i) a separate set of genes for blue monochromasy at this locus and (ii) a defect in both red and green pigment gene structure. In accordance with this notion, it has been recently found [104] that patients carrying blue cone monochromasy exhibit alterations in both red and green pigment gene clusters. Based on the mutational studies two pathways to blue cone monochromasy have been suggested [104]. One of these involves a homologous unequal recombination followed by inactivation by mutation. The second pathway consists of deletions of the sequences in the upstream of the red pigment gene resulting in the loss of both red and green gene functions. In this respect blue cone monochromasy is very similar to β^{o}-thalassemia which also involves deletion of upstream sequence and loss of function of more than one gene.

XII. Familial exudative vitreoretinopathy (FEVR)

This disorder, which was first reported by Criswick and Schepens [105], is a new entry to the X-linked ocular diseases [106]. FEVR is a congenital hereditary bilateral disease which affects both retina and vitreous body. The disorder (both autosomal and X-linked) is characterized by the abnormal vascularization of the peripheral retina and affects both eyes with nearly 100% penetrance. More severely affected eyes show a variety of signs such as ectopia of the macula, retinal traction, sub- and intraretinal exudates, retinal fold or retinal detachment and vitreous hemorrhage [107]. It is a slowly progressive disease but ultimately can lead to blindness from retinal detachment. The condition is clinically similar to retinopathy of prematurity [108], but the affected individuals do not have a history of premature birth or oxygen therapy and have a normal gestational period. This disease is very widely known to be transmitted through an autosomal dominant mode and hence often referred to as dominant exudative vitreoretinopathy [109,110]. However, recent evidence

TABLE I

Map location of several X-linked genetic eye disorders described in the article

Disorders	Map location	Ref.
1. Ocular albinism OA1	Xp22.3-p22.2	12
Ocular albinism OA2	Xp21.3-p21.2	11
2. Retinoschisis	Xp22.2-p22.1	17, 18
3. Cataract (Nance-Horan)	Xp22.3-p21.1	19, 23, 24
4. Retinitis pigmentosa 3	Xp21	26, 33
Retinitis pigmentosa 2	Xp 11.3-p11.2	26, 27
5. Norrie disease	Xp11.3-p11.2	37, 39, 43, 44
6. Congenital stationary night blindness	Xp11.3	59–63
7. Choroideremia	Xq21.2	67–71
8. Fabry disease	Xq21.33-q22	77, 78
9. Lowe Oculocerebrorenal Syndrome	Xq24-q26	86–88, 90–92
10. Color blindness, deutan, protan and blue cone monochromacy	Xq28	93–97

TABLE II

Map location of X-linked eye disorders which are not discussed in the article

Disorder	Map location	Ref.
1. Kallman syndrome	Xpter-p22.32	112, 113
2. Aicardi syndrome	Xp22.3-p21.3	114
3. Corneal dermoids	Xp22.2-p22.1	115
4. Progressive cone dystrophy	Xp21.1-p11.3	116
5. Menkes disease	Xq12-q13	117
6. Megalocornea	Xq12-q26	118, 119
7. Aarskog syndrome	Xq13	120
8. Alport syndrome	Xq22-q24	121, 122
9. Incontinentia pigmenti	Xq27-q28	123, 124
10. Hunter syndrome	Xq27-q28	125
11. Anophthalmos	Xq27-q28	126
12. Myopia	Xq28	127

has been accumulated for an X-linked mode of inheritance [106]. Preliminary linkage analyses (our unpublished results) suggest the possibility of multiple candidate loci as putative location of the genes. These studies once again may reflect that FEVR may belong to a group of genetically heterogeneous hereditary disorders of the retina. Use of several other DNA markers for the long and short arm of the X-chromosome may accelerate the linkage analysis of this disorder.

XIII. Concluding comments

Modern medicine is already being affected by the advances in recombinant DNA technology. Increasingly, many genetic diseases (mostly single gene diseases) have been diagnosed before the onset of the related syndrome and many more will be detected in the near future. The ocular field is not an exception to this exciting progress. During the last decade, X-linked choroideremia, Norrie disease and color blindness genes have been isolated and characterized, helping investigators to understand the molecular basis of these hereditary disorders. In addition, several other diseases such as retinitis pigmentosa, retinoschisis, cataracts and ocular albinism disease have been mapped to a particular region of the X-chromosome (Tables I and II). In the next decade, undoubtedly many of these genes will be cloned, characterized and their molecular pathology defined. Although carrier detection, prenatal diagnosis and gene replacement therapy have not so far been extensively tried for the genetic diseases of the ocular systems, such attempts will be now feasible [111]. Molecular analyses made it clear that there are many challenging problems that need attention. For instance (i) we need to understand more about the altered gene products; (ii) it is necessary to know the relationship between the pathology and gene alteration; (iii) it is necessary to establish the primary cause that is responsible for the disorder; (iv) it is essential to discover why certain disorders have such heterogeneous phenotypes; (v) we need to improve the quality of genetic counseling to prepare the families for a difficult educational job; (vi) we need to gain new knowledge related to retinal and choroidal function; and (vii) it is necessary to develop a new approach to clinical diagnosis. Additionally, generation of the transgenic animals with the defective genes may provide better insight into the pathogenetic mechanisms of these disorders which may in turn provide better therapy. This will also help to understand why certain disorders have such heterogeneous phenotypes and improve the quality of genetic counseling. Gene targeting by homologous recombination may prove to be another avenue of approach to understand the functions of genes and complexities of multigenic diseases. These approaches will hopefully help to meet the ultimate challenge of molecular biology in identifying the individuals at risk, defining the clinical phenotype at the molecular level, understanding of the functions of the genes, reversing the genetic defects and restoring sight to the blind.

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While this paper was in press, further studies (Bergen, A.A.B., Zijp, P., Schuurman, E.J.M. et al. (1993) Genomics 16, 272–273; Charles, S.J., Green, J.S., Moore, A.T. et al. (1993) Genomics 16, 259–261) on ocular albinism (OAI) have shown that OAI has the following gene map: Xpter-STS-DXS237-KAL-(OAI, DXS143)-DXS85-DXS16-Xcen., which is consistent with the previous report [12]. Furthermore, analysis of genomic clone encompassing the Norrie disease gene indicates that the gene is not only expressed in the eye but also in the fetal and adult brain, lung and muscle (Chen, Z.-Y., Battinelli, E.M., Hendriks, R.W. et al. (1993) Genomics 16, 533–535.

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Mitochondrial Diseases: Gene Mapping and Gene Therapy

Minireview

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The human mitochondrial genome is a 16,569 bp circular DNA molecule that is strictly maternally inherited and encodes 13 of the proteins required for oxidative metabolism, as well as 22 tRNAs and 2 rRNAs required for their translation. Because mitochondrial DNA has much less redundancy than the nuclear genome (in which essentially identical information is received from both parents, and tRNAs and rRNAs are present in multiple copies) and much higher information density (an equivalent complement of essential nuclear genes might be strewn across 1–2 million bases), it would seem to be an excellent target for mutations giving rise to human disease. Discoveries from a number of labs have confirmed this expectation:

- Leber's hereditary optic neuropathy (LHON) had long been suspected to be caused by a mitochondrial mutation owing to its maternal inheritance pattern. By sequencing mitochondrial DNA from LHON patients and comparing it with the canonical Cambridge sequence, Wallace et al. (1988) identified in LHON patients a missense mutation in a conserved position in subunit 4 of NADH:ubiquinone reductase (ND4). Seven other observed nucleotide changes appear to be polymorphisms.
- Similarly, a neurological syndrome consisting of retinitis pigmentosa, ataxia, seizures, dementia, and proximal muscle weakness is caused by a missense mutation in a conserved position of subunit 6 of the mitochondrial ATPase complex (Holt et al., 1990).
- Many patients with an encephalomyopathy called Kearns-Sayre syndrome bear large, heterogeneous deletions in the mitochondrial chromosome (e.g., Holt et al., 1988; Ozawa et al., 1988; Rötig et al., 1988; Zeviani et al., 1988). To account for the relatively homogeneous phenotype produced by the various deletions, investigators argue that virtually any deletion of at least 1–2 kb will eliminate a tRNA gene essential for mitochondrial translation. But in view of the identification of Kearns-Sayre patients bearing large duplications (Poulton et al., 1989), this is not a wholly adequate explanation.
- Finally, myoclonic epilepsy associated with ragged red muscle fibers (MERRF) is caused by a single nucleotide substitution in the TψC loop of the $tRNA^{Lys}$, which apparently interferes with mitochondrial translation (Shoffner et al., 1990).

In most of the diseases, patients' cells carry a mixture of mutant and normal mitochondria—a condition known as heteroplasmy—with the proportions varying from tissue to tissue and from individual to individual within a pedigree, in a manner roughly correlating with the severity of the phenotype. Each tissue functions like a chemostat with two species of bacteria competing, but with a perverse twist: lesions in genes essential for oxidative metabolism may cause no growth disadvantage, because all factors required for mitochondrial growth and replication are encoded by the nuclear genome and imported from the cytoplasm. Conceivably, some mutations might even confer a selective advantage on the mutant mitochondria—if large deletions of DNA permit more efficient replication (Wallace, 1989), or if inadequate function actually provokes mitochondrial replication (Grossman, 1990).

It is instructive to compare the genetic mapping of diseases encoded by nuclear genes versus mitochondrially encoded ones. To map a nuclear gene responsible for a simple Mendelian trait, the inheritance pattern of the disease is compared with the inheritance pattern of a collection of DNA polymorphisms distributed throughout the genome. For each polymorphic marker, one computes the likelihood ratio, which consists of the probability that the inheritance pattern would result if the disease and marker are linked, divided by the probability that the pattern would result if they are unlinked. Linkage is said to be proven if the likelihood ratio is large enough; traditionally, the $\log_{10}$ of the likelihood ratio (called the LOD score) must exceed 3. A total of 10 informative meioses is needed to reach a LOD score of 3. Once linkage is established, the genetic position of the disease-causing mutation can be determined by recombinational mapping relative to a variety of nearby markers.

By contrast, mitochondrial gene mapping requires no DNA polymorphisms. One need only show that a disease exhibits transmission through all mothers and no fathers in a sufficiently large family. Finer recombinational mapping is impossible because mitochondria from two parents cannot be brought together and persuaded to recombine. Once linkage is established, one must sequence the mitochondrial chromosome and identify the mutation from among a background of harmless polymorphisms. (The contrast between the nuclear and mitochondrial genomes is actually less pronounced in practice than in theory. Owing to the limited number of meioses that can be studied in human families, recombinational mapping of nuclear chromosomes rarely has a resolution finer than 100,000 bp, and one must resort to scanning a large stretch of DNA sequence.)

Although DNA sequence can provide strong evidence supporting the assignment of a mitochondrial disease to a particular mutation, such data are not conclusive (unless the entire mitochondrial genome were sequenced and only a single nucleotide difference observed). Rigorously, the functional significance of a mitochondrial DNA mutation must be proven by showing that the introduction of the wild-type gene can complement the defect. Unfortunately there is, as yet, no suitable transformation system for reintroducing mitochondrial genes into the organelle.

There are, however, two examples in yeast where a mitochondrial gene has been introduced into the nuclear genome, and the resultant protein is transported into the mitochondrion and is functionally active (Nagley et al., 1988; Hartl and Neupert, 1990). A similar approach could

be used for the LHON mutation in ND4. To understand how this might be done, it is necessary to review briefly how mitochondrial proteins reach their final destinations.

Biogenesis and membrane insertion of mitochondrially encoded proteins are not well understood, but one or more signal sequences on each of these proteins is likely to target them from their site of synthesis on mitochondrial ribosomes in the matrix space to the appropriate inner membrane location. Indeed, physiological and molecular studies on yeasts with mitochondrial DNA mutations in ATP synthase subunits have shown that certain subunits are essential for the ordered insertion of others (Nagley, 1988).

Nuclear-encoded proteins destined for the mitochondrial matrix or inner membrane are synthesized on cytosolic ribosomes; with few exceptions they contain an N-terminal mitochondrial matrix–targeting sequence that is cleaved by a matrix protease after uptake into the organelle matrix space. Such sequences are thought to bind to one or more receptors on the mitochondrial outer membrane. Proteins destined for the inner membrane are believed to cross both the outer and inner membranes into the matrix space and then become inserted into the inner membrane.

In one study, a S. cerevisiae mitochondrial gene encoding the hydrophobic subunit 8 of the F_0F_1 synthase was modified by addition at the N-terminus of a cleavable matrix-targeting sequence from a nuclear-encoded ATP synthase subunit. When introduced into the yeast nucleus, this modified gene directed synthesis of a subunit 8 protein that was correctly incorporated into the mitochondrion, and complemented a yeast mitochondrial DNA mutation in the subunit 8 gene (Nagley et al., 1988). Similarly, Hartl and Neupert (1990) showed that the mitochondrially encoded precursor of Neurospora cytochrome oxidase subunit II (preCOXII) could be synthesized on cytosolic ribosomes and imported into Neurospora mitochondria, provided that a matrix-targeting sequence was joined to its N-terminus. The matrix-targeting sequence was correctly removed, and the resultant preCOXII, identical to the normal mitochondrially encoded precursor protein, was subsequently processed and targeted to its correct submitochondrial location.

Although similar successful studies on relocation of human mitochondrial genes to the nucleus have not been reported, cultured cells from patients with the LHON mutation, and defective in oxidative phosphorylation, would be ideal recipients for such gene replacement. A functional mitochondrial ND4 protein, encoded by a nuclear gene and modified to contain a matrix-targeting sequence, could be det cted by complementation of the defect in oxidative phosphorylation.

A similar gene-replacement th rapy for a mutant mitochondrially encoded tRNA might s em mor farf tched. However, import of nucl ic acid into mitochondria has been report d. Vestweber and Schatz (1988) chemically coupl d a single- or double-strand d 24 bp piece of DNA to a (nuclear-encod d) mitochondrial precursor protein. Th y show d that the DNA–prot in compl x was imported into the yeast mitochondrion and, in most cases, the matrix-targeting sequence was r moved.

More relevant though, are the studies of Chang and Clayton (1989, and references therein) on a mitochondrial processing endonuclease that is essential for cleavage of a particular mitochondrial RNA in one step of mitochondrial DNA replication. The enzyme is a ribonucleoprotein whose 136 base RNA component is a nuclear gene product. The single-copy gene encodes a 275 base uncapped primary transcript that is subsequently processed. How the RNA is imported into the mitochondrion and where the 5′-most 139 bases are removed are not known. However, either the RNA itself or a protein to which it is bound presumably contains some mitochondrial targeting information. Possibly additional sequences of ribonucleotides linked to the 236 base precursor are also taken up into the organelle. We note that all mitochondrial tRNAs are generated by cleavage of long RNA precursors that also encode mitochondrial mRNAs or rRNAs. Thus as an example, the mitochondrial $tRNA^{Lys}$ flanked by these cleavage sequences and linked to the 236 base precursor of the endonuclease RNA might be correctly processed after uptake into the mitochondrion.

Lest one become too optimistic about the possibilities of gene therapy for diseases caused by mutations in mitochondrial DNA, it must be pointed out that the major affected tissues, such as optic neurons and striated muscle, contain nondividing cells. Myoblast nuclei, for instance, cease DNA synthesis before the cells fuse to form a multinucleate syncytium. Vectors for generation of stable transformants of mammalian cells, such as retroviruses, require DNA replication for insertion into genomic DNA. Thus, if gene therapy for these diseases is to be considered, it may require development of vectors that can be retained and expressed in nondividing cells.

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In Vivo Transfer of a Reporter Gene to the Retina Mediated by an Adenoviral Vector

Tiansen Li, Michael Adamian,* Dorothy J. Roof,* Eliot L. Berson,* Thaddeus P. Dryja,* Blake J. Roessler,† and Beverly L. Davidson†*

Purpose. The ability of replication-deficient adenovirus to mediate gene transfer to retinal c[illegible] was evaluated.

Methods. A replication-deficient adenoviral vector, AdCMVβA.*ntlacZ*, which contains the bacterial β-galactosidase (*lacZ*) reporter gene, was injected into the subretinal space of normal, *rd*, and *rds* strains of mice at various ages. The efficiency and duration of transgene expression were assessed by histochemical examination and transmission electron microscopy.

Results. AdCMVβA.*ntlacZ* was effective in mediating gene transfer to the retinal pigment epithelial cells, rod and cone photoreceptor cells, and cells in the inner nuclear layer of the retina for periods of up to 1 month. Gene transfer to retinal pigment epithelial cells occurred at much lower viral titers than was required for gene transfer to photoreceptor cells. The extent to which photoreceptor cells could be transduced varied with the age of the animals and the conditions of the photoreceptor cells: greater numbers of photoreceptor cells were transduced in 5- to 7-day-old pups and in mice at the initial stages of photoreceptor degeneration than in normal adult mice. No evidence of gross pathogenic effects or viremia in recipient mice was observed.

Conclusions. Replication-deficient adenovirus mediates transfer and expression of a foreign gene in retinal pigment epithelial and photoreceptor cells. Gene transfer to photoreceptor cells is enhanced in developing retinas or at the predegenerate stage of photoreceptors in genetically programmed retinal degeneration. Invest Ophthalmol Vis Sci. 1994;35:2543–2549.

Retinitis pigmentosa (RP) is the name given to a group of inherited retinal degenerative diseases. Mutations in three genes—opsin, the β subunit of rod cGMP phosphodiesterase, and peripherin/*rds*–have thus far been identified to cause RP.[1–5] Each of these genes is expressed primarily or specifically in the photoreceptor cells. In the *rd* and *rds* mouse models for RP, which carry *null* alleles of the genes for the β subunit of rod cGMP phosphodiesterase and peripherin, respectively, germline introduction of functional gene constructs rescued the photoreceptor cells from genetically programmed degeneration.[6,7] This raises the possibility that specific and local in vivo introduction of genes into the photoreceptor cells as a replacement for certain mutant alleles could potentially be a viable approach to treatment. Such gene replacement therapy for recessive RP, as well as potential therapies aimed at alleviating the effects of dominant RP alleles, such as antisense, antigene, or ribozyme strategies,[8–11] must have as a prerequisite the ability to deliver genetic elements to retinal cells in vivo.

Physical, chemical, and biologic methods have been evaluated for in vivo transfer of foreign genes into somatic tissues. Among these, the use of gene transfer vectors derived from retroviruses proved to be effective in mediating stable gene transfer to a wide range of tissues, but only if the target cells were undergoing cell division. However, cells in the adult neural retina are postmitotic and are therefore not receptive to retrovirus-mediated gene transfer. Replication-deficient herpes simplex virus 1 (HSV-1) may hold promise for gene transfer to postmitotic neurons,[12] but the current versions of HSV-1–based vectors, most of which have a singl immediat early gene inactivated, are cytotoxic.[13] In comparison, replicati n-deficient

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adenoviruses are less cytotoxic and can be prepared and delivered to tissues in vivo at high titers resulting in high levels of gene transf r without sev re tissue damage.[14–16] Recent work on ad novirus-mediated gene transfer to the brain[17–19] encourages the idea that adenoviral vectors may also be useful for gene transfer to the neural retina. In the present study we explored the use of replication-deficient adenovirus as a means to transfer a foreign gene to retinal cells in normal mice as well as in the *rd* and the *rds* mice. Our data suggest that adenoviral vectors are useful for somatic gene transfer to the retina.

METHODS

Animals

The C.B-17 mice, which differ from the Balb/c strain only at the Igh-1 locus, were obtained originally from Charles Sidman (Jackson Laboratories, Bar Harbor, ME). The C.B-17 mice suffer no retinal degeneration. The FVB/n mice homozygous for the *rd* allele were obtained from Jackson Laboratories. The homozygous *rds* mice in a Balb/c background were provided by Richard Sidman and Macy Tang (New England Primate Center). Animals were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Preparation of Viral Suspension for Injection

The method for adenovirus preparation was modified from that described by Graham and van der Eb.[20] The construction of AdCMVβA.*ntlacZ* was essentially as previously described except that the nuclear localization sequence from the SV40 large T antigen was fused to the 5′ end of the *lacZ* gene.[21] AdCMVβA.*ntlacZ* were amplified on permissive 293 cells[21] (obtained from American Type Culture Collection, [ATCC] CRL 1573) in Dulbecco's modified Eagle medium supplemented with 10% bovine calf serum. Subconfluent cells were infected with AdCMVβA.*ntlacZ* at 10 plaque-forming units (pfu)/cell, harvested 30 hours later and the virus purified by CsCl gradient centrifugation. Desalting was then carried out by column chr matography using a Sephadex G-25 column (NAP-10 column, Pharmacia) equilibrated with minimal essential medium. The efflu nt containing the virus was centrifuged through a Centricon-100 concentrator (Amicon) to reduce the volume to about 100 μl. Viral titers of the concentrated stock were typically between 10^{11} to 10^{12} pfu/ml. Fresh virus preparations were used immediately afte - dilution in minimal essential medium for subretina' injections.

In Vivo Delivery

Mice were anesthetized by intraperit neal injection of sodium pentobarbital. Subretinal i ctions were performed under an ophthalmic s gical microscope. After making a incision slightly be ind the ora serrata with a microscalpel, a Hamilton yringe with a 1.3-cm/33-gauge blunt-ended needle was inserted tangentially toward the back of the eye. Approximately 0.3 to 0.5 μl of viral suspension was injected per eye. Control eyes were injected with an equal volume of minimal essential medium. Proper delivery into the subretinal space was confirmed by the appearance of a partial retinal detachment seen by indirect funduscopy. Intravitreal injection was performed similarly except that the injection needle was inserted vertically through the neural retina.

Eight adult C.B-17 (normal) mice, 30 C.B-17 pups (age 5 to 7 days), 10 *rd* pups (age 7 days), and 3 adult *rds* mice (age 2.5 months) were injected subretinally with purified AdCMVβA.*ntlacZ* at high titer (10^{11} pfu/ml). This is the highest titer we could routinely obtain in viral preparations. Two 7-day-old C.B-17 pups were injected subretinally with the virus at lower titer (10^{8} pfu/ml), and 2 C.B-17 adult mice were injected intravitreally with the low titer virus (10^{8} pfu/ml). Control mice were injected witl viral suspension medium. Indirect funduscopy after subretinal injection of 0.5 μl showed a partial retinal detachment over approximately one quarter of the retinal area in adult mice (Fig. 1), allowing exposure of retinal pigment epithelium (RPE) and the neural retina to the injected material. This partial retinal detachment usually disap-

FIGURE 2. Histochemical examination of retinas injected with AdCMVβA.*ntlacZ*. Transduced cells expressing the lacZ reporter gene can be readily identified by their blue appearance. Representative sections from mice sacrificed 1 to 6 weeks postinjection, counterstained with neutral red to show layers of retinal structure. RPE, retinal pigment epithelium; ONL, outer nuclear layer; INL, inner nuclear layer. (a) Near-uniform labeling of RPE but no photoreceptor cells in an adult C.B-17 mouse retina 1 week after injection. ×1600. (b, c) Gradual reduction in the number of *lacZ*-positive cells in C.B-17 mouse retinas injected at 5 to 7 days of age and examined at 4 (b) and 6 (c) weeks. ×1600. (d) Abundant labeling of both RPE and photoreceptor cells in an *rd/rd* mouse retina injected at 7 days of age and examined at 1 week postinjection. ×400. (e) Labeling of photoreceptor cells in an *rds/rds* mouse retina injected at 2.5 months of age and examined 1 week later. Cells in the inner nuclear layer are also labeled. ×1600. (f) Labeling of the corneal endothelium and the iris pigment epithelium in an adult C.B-17 mouse retina injected intravitreally and exa ined 1 week later. ×400.

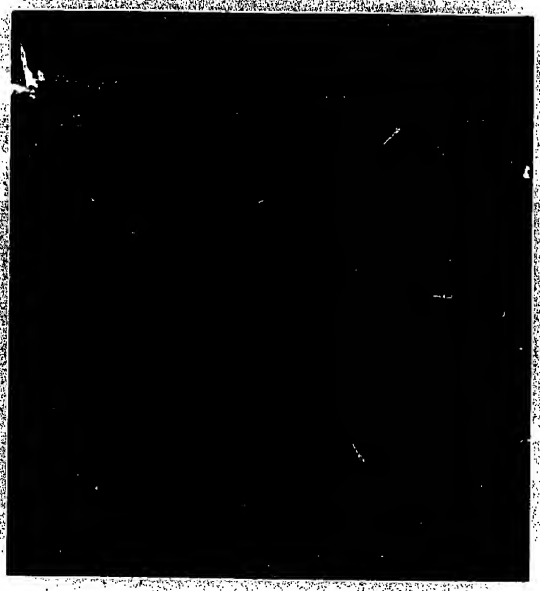
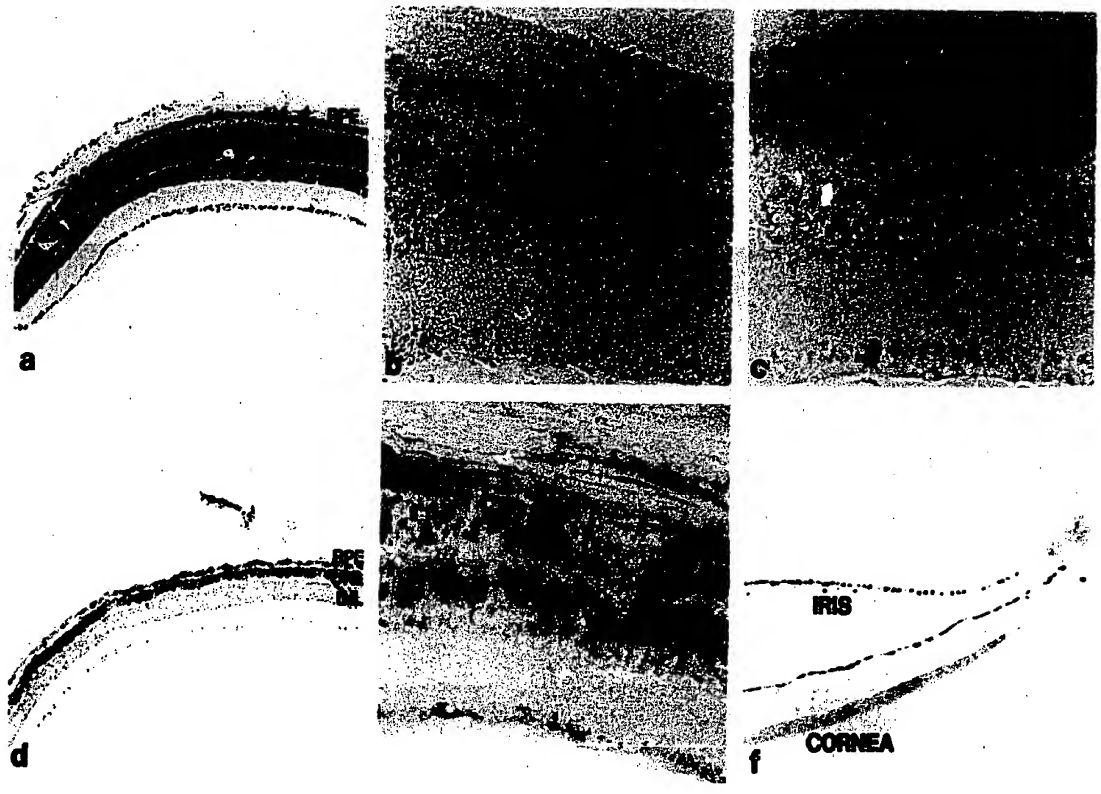


FIGURE 1. A fundus photograph showing partial retinal detachment after subretinal injection of 0.5 μl. Arrowheads delineate the area of detachment. Shown here is a normal adult pigmented mouse retina.

peared within 3 days. In all cases one eye of each mouse was left uninjected to serve as an internal control.

Histochemical Examination

Normal pups were euthanized and eyes were enucleated and processed for histochemical examination at 1, 4, 6, or 8 weeks postinjection. The *rd*, *rds*, and normal adult mice were analyzed at 1 week postinjection. Eyes were fixed in 0.5% glutaraldehyde in phosphate-buffered saline for 1 hour at room temperature. The anterior segments and the lens were removed, and the eye cups were incubated at room temperature for 2 hours in a solution of 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 1 mg/ml 5-bromo-4-chloro-3-indolyl-b-D-galactoside (X-gal) in phosphate-buffered saline at pH 7.8. After staining for *lacZ* activity, the eye cups were postfixed in 2.5% glutaraldehyde and 1% formaldehyde in 0.1 M sodium cacodylate buffer (pH 7.0) for 1 hour or longer, embedded in acrylamide,[22] frozen in OCT compound, and cryosectioned at 12- to 18-μm thickness. Sections were examined either unstained or counterstained with neutral red.



Electron Microsc py

Histochemically stained *rd* mouse eye cups w re post-fixed in 2.5% glutarald hyd and 1% formaldehyde in 0.1 M sodium cacodylat buffer for 1 hour, followed by 1% osmium tetroxide in 0.1 M sodium cacodylate buffer. The eye cups were then washed a:id dehydrated in a graded series of ethanol solutions and embedded in Spurr's medium. Ultrathin sections (0.1 μm) were cut and stained with uranyl acetate and lead citrate. The sections were then viewed under a JEOL 100C electron microscope.

RESULTS

In Vivo Gene Transfer to Retinal Cells

At 1 week postinjection of AdCMVβA.*ntlacZ*, gross inspection of the eye cups after histochemical staining with X-gal showed *lacZ* activity in an area encompassing 25 to 50% of the retina. Light microscopic examination of multiple serial sections from this area demonstrated *lacZ* gene expression (blue-staining nuclei) in several retinal cell types, including RPE cells, photoreceptor cells, and to a lesser extent, cells in the inner nuclear layer (Figs. 2a to 2e). Control eyes injected with suspension medium or eyes left uninjected were all negative for *lacZ* activity. Throughout the blue-staining areas in viral injected eyes, *lacZ*-positive RPE cells were close to one another, indicating nearly 100% of the RPE cells were transduced (Figs. 2a, 2d). Adenovirus-mediated gene transfer to RPE was reproducible in mice of different strains and ages. Gene transfer to photoreceptor cells, however, was most efficient in 5- to 7-day-old pups and in mice whose photoreceptor cells were in the early stage of degeneration (Figs. 2d, 2e). Comparatively, *lacZ* staining of photoreceptor cells in normal adult retina was either absent or limited to very small areas where the retinal structures were apparently injured by the injection needle (Fig. 2a; additional data not shown). The regions occupied by *lacZ*-positive photoreceptor cells and *lacZ*-positive RPE cells essentially overlapped, with the latter consistently occupying a wider area. However, in the center of most abundant photoreceptor cell labeling, there were fewer and sometimes no labeled RPE cells, with positive RPE cells increasing in abundance away from this region.

To determine whether RPE cells and photoreceptor cells could be transduced at lower multiplicity of infection, a low titer viral suspension (10^8 pfu/ml) was injected subretinally in 7-day-old normal pups. Upon examination 1 week postinjection, we found near uniform labeling of the RPE cells but no labeling of pho- t receptor cells. As a comparison, injection of the virus at 10^8 pfu/ml intravitreally resulted in efficient gene transfer to the corn al endothelium and the iris pigment epithelium (Fig. 2f) and retinal ganglion c lls, but not to cells in the inner and outer nuclear layers of the retina (data not shown).

The percentage of *lacZ*-positive cells of all types decreased over time. In normal mice injected at 5 to 7 days of age, the abundance of labeled photorec ptor cells at 1 week postinjection was simil..r to injected *rd* retina but decreased on average to ess than half of that at 4 weeks postinjection (Fig. 2' . At 6 weeks postinjection, only few and scattered abeled cells were seen (Fig. 2c).

Electron Microscopy Analy is

Precipitates of X-gal reactior product are electron dense and can be visualized using transmission electron microscopy.[17] To determine whether both rod and cone photoreceptor cells could be transduced by AdCMVβA.*ntlacZ*, EM analysis was done on histochemically stained *rd* mouse retinas injected with AdCMVβA.*ntlacZ* 7 days earlier. X-gal precipitates were evident in both rod and cone photoreceptor cells (Figs. 3a, 3b) and in RPE cells (Fig. 3b).

Pathogenicity of Subretinally Administered AdCMVβA.*ntlacZ*

Photoreceptor outer segments were shorter or absent in the areas exposed to the virus when examined at 1 week postinjection. There was no apparent infiltration by inflammatory cells. In adjacent areas not exposed to the virus, the retinal structures appeared indistinguishable from uninjected control eyes. Some of these local pathologic changes could be attributed to the effect of retinal detachment, as control eyes injected with suspension medium also displayed some shortening of outer segme its, although to a lesser extent. The absence of labeled RPE cells in some regions with abundant labeled photoreceptor cells might reflect acute cell death due to high multiplicity of infection and subsequent replacement with replicating adjacent RPE cells. Retinas examined at 8 weeks postinjection showed a full complement of retinal cell layers and inner and outer segments. In some sections, however, there were areas with reduced thickness of the outer nuclear layer and shortened inner and outer segments compared to adjacent areas.

There were no fatalities due to subretinal administration of AdCMVβA.*ntlacZ*. The development of mouse pups injected with AdCMVβA.*ntlacZ* appeared similar to that of uninjected littermates. At the time of sacrifice, the injected eyes were of comparable size to the contralateral control eyes. To determine if the ad - novirus could spread outside of the eyes, serum samples were taken at 1 and at 4 weeks aft r injection and inoculated onto cultures of 293 cells. The cultures were serially passaged 5 tim s and did not show any

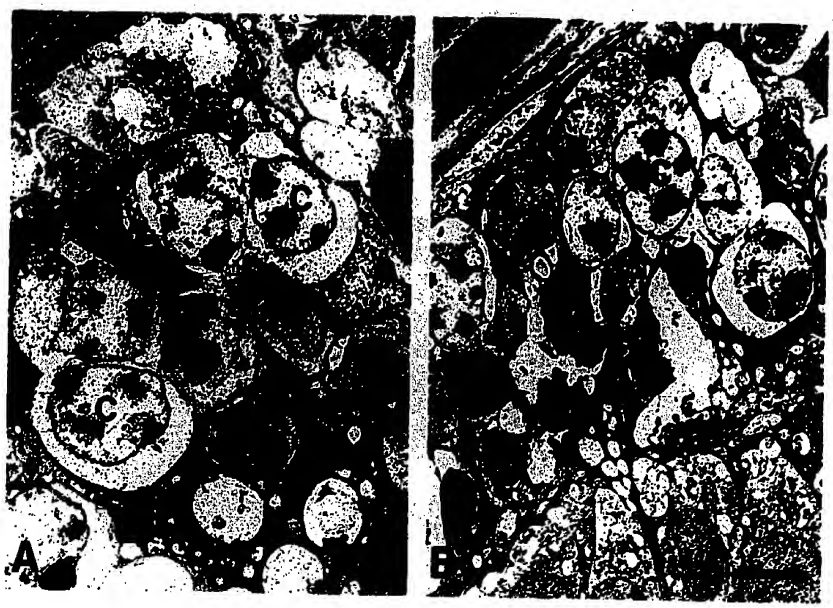


FIGURE 5. Transmission electron microscopy analysis of an X-gal–stained retina. Shown is a section of FVB/n *rd/rd* mouse retina injected with AdCMVβA.*ntlacZ* at 7 days of age, sacrificed and processed for *lacZ* activity 7 days later. *LacZ*-expressing cells are identified by electron-dense particles concentrated around their nuclear membranes. (a) Rod and cone photoreceptor cells are distinguishable by their heterochromatin patterns. Representative cone (C) and rod (R) photoreceptor cells containing X-gal precipitate. (b) A RPE cell intensely labeled with *lacZ* reaction product. A pyknotic cell is seen (arrow). Scale bar, 10 μm.

evidence of viral infection. Mouse brain, lungs, and liver tissues taken at the time of sacrifice and histochemically stained for *lacZ* activity were negative for X-gal–stained cells. These data suggest that adenovirus injected into the subretinal space does not readily spread through systemic circulation.

DISCUSSION

In the current study, we have demonstrated that a recombinant human adenovirus can mediate the transfer and expression of a foreign gene to retinal cells under conditions that cause neither a severe disruption of retinal structure nor any apparent systemic toxicity. Importantly, both rod and cone photoreceptor cells are receptive to gene transfer, as are RPE cells. Photoreceptor cells are currently the primary target of gene transfer, because the mutant genes causing RP identified so far are all expressed primarily in the photoreceptor cells. However, given the close dependence of photoreceptor structure and function on RPE cells, future research may reveal a role for RPE cells in some forms of RP, thus making them a target for gene transfer as well. Targeting RPE cells with recombinant adenovirus has the advantage that a lower viral titer can be used, resulting in even lower cytotoxicity and higher efficiency of gene transfer.

In these experiments adenovirus-mediated gene transfer compares favorably with HSV-1–derived vectors with respect to efficiency of photoreceptor cell transduction and pathogenicity. In an earlier set of experiments, the efficiency of HSV-1 gene transfer to photoreceptor cells was consistently low, possibly because of the inability to purify HSV-1 to very high titers.[23] Furthermore, even at the relatively low titer of 10^7 to 10^8 pfu/ml, there were gross pathogenic effects as evidenced by failure of eye development in some cases and neurologic symptoms after subretinal injection in young pups (ref 23 and unpublished data). Thus, currently, adenovirus-mediated gene transfer appears to be technically superior to HSV-based vectors for the introduction of foreign genes into the retina.

In vivo gene delivery to photoreceptor cells presents a significant challenge relative to other cell types in the eye. In normal adult mice, subretinal injection of AdCMVβA.*ntlacZ* at 10^{11} pfu/ml results in essentially 100% transduction of RPE cells, with no gene

transfer to photoreceptor cells. In 5- to 7-day-old pups and in mice carrying mutations that cause retinal degeneration, however, AdCMVβA.*ntlacZ* readily transduced photoreceptor cells. In contrast, RPE cells, the corneal endothelium, and the iris pigment epithelium were efficiently transduced at a titer of 10^8 pfu/ml, and the transfer efficiency was not related to age or mutations affecting the retina. One common feature shared by the developing retina and pre-degenerate mutant mouse retina is that the photoreceptor outer segments are shorter or absent. This suggests that the long, densely packed outer segments and/or the healthy interphotoreceptor matrix in normal adult mouse retina may act as a physical barrier to virus-mediated gene transfer.

The number of cells in the retina expressing the reporter gene decreased over time, as has been reported for similar adenovirus-mediated gene transfer experiments in other tissues. Adenovirus DNA reportedly remains episomal in infected cells, with the loss of transgene expression due to loss of transferred DNA or its inactivation without DNA loss. Alternatively, the possibility that some transduced cells were lost because of immune reactions against transduced cells could not be excluded. Thus, for practical gene therapy applications, vectors need to be generated that will increase the stability of transgene expression.

In summary, our data demonstrate a novel method for gene transfer to the photoreceptor cells in developing mice and in mouse models of RP. Thus, recombinant adenovirus containing the complementary DNAs for the β subunit of rod cGMP phosphodiesterase or peripherin may be effective in rescuing photoreceptor degeneration after transduction into the *rd* and *rds* mice, respectively. Such investigations should provide direct information on the feasibility of this approach to treating inherited retinal diseases in humans. Additionally, these techniques should be generally applicable to basic research into retinal cell physiology and the regulation of photoreceptor cell-specific gene expressions.

Key Words

adenoviral vector, viral transduction, gene transfer, gene therapy, retinitis pigmentosa

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2098

ANALYSIS BY ILLEGITIMATE TRANSCRIPTION OF A MUTATION IN THE 5' SPLICE SITE IN EXON 8 OF THE PAH GENE. L. R. Desviat, B. Pérez & M. Ugarte. Centro de Biología Molecular "Severo Ochoa" CSIC-UAM. Cantoblanco 28049 Madrid. SPAIN.

Up to now, 12 splice defects have been described within the PAH gene. Using PCR-SSCP and sequence analysis we have found a point mutation involving the last nucleotide in exon 8 (CAG/ CAA). The G to A substitution does not alter the aminoacid (Q304Q), but it may cause a splice defect, as it is included in the 5' splice donor site, and the G at this position is highly conserved (80%) in all eukaryotic genes. We have analysed by illegitimate transcription the PAH mRNA in lymphocytes of a patient bearing the mutation in heterozygous fashion. After RT-PCR we observed once the appearance of an extra larger band, which could be due to the use of a cryptic splice site instead of the mutated one. Furthermore, sequencing of 6 clones of the band of expected size in the patient revealed that all had the normal sequence, in spite of the G to A substitution being found in the genomic DNA. In view of these results, we believe that the larger extra band represents the allele with the mutation which causes a highly unstable mis-spliced RNA. This splice defect could be, therefore, the disease causing mutation in the patient.

2099

Isolated respiratory chain enzyme deficiency in patients with a mitochondrial (encephalo-) myopathy: sequence analysis of the mitochondrial complex I and IV genes. D. de Vries[1], J. de Coo[1], P. Buddiger[1], W. Ruitenbeek[2], R. Albrecht[3] and B. van Oost[1]. [1]Department of Human Genetics, University Hospital, Nijmegen, The Netherlands, [2]Metabolic Division, Department of Pediatrics/Neurology, University Hospital, Nijmegen, The Netherlands, [3]Zentrum für Humangenetik, Universität Bremen, Bremen, Germany.

The mitochondrial respiratory chain consists of four enzyme complexes. Deficiencies of complex I (NADH dehydrogenase) and complex IV (cytochrome c oxidase) are frequently found in muscle biopsies from patients with a mitochondrial (encephalo-)myopathy. Mutations in the mitochondrial encoded subunits have been observed in a number of different mitochondrial (encephalo-)myopathies. We screened eight mitochondrial (encephalo-)myopathy patients with an isolated complex I deficiency for mutations in the ND genes by direct sequencing. No abnormality was detected. We also studied 9 mitochondrial (encephalo-)myopathy patients and an isolated complex IV deficiency. In the muscle biopsy of one patient a novel heteroplasmic mutation (T → C) at nucleotide position 6681 was found in the mitochondrial COX I gene. This mutation led to the substitution of a conserved Tyr for His. As this mutation changed the secondary structure of the protein and was not found in the healthy mother, we consider it likely that this mutation is pathological. In the other patients no abnormality was detected. Therefore, mutations in the mitochondrially-encoded subunits are not a frequent cause of isolated respiratory chain enzyme deficiency.

2100

A new agarose matrix for single-strand conformation polymorphism (SSCP), heteroduplex (HTX), and gel shift analyses. ((M. M. Dumais[1], H. W. White[1], M. R. Rashid[1], J. Choi[2], and G. Ruano[2].)) [1]FMC BioProducts, Rockland, ME, and [2]BIOS Labs., New Haven, CT. Intro. by: Ivan Balazs

Detection of mutation, by SSCP or heteroduplex analysis, is important in medical genetics and oncology. Analysis of DNA binding proteins is a powerful tool in molecular biology research. Traditionally, these methods are performed using nondenaturing gel electrophoresis on polyacrylamide or polyacrylamide-type matrices. Here we report the development of a new agarose gel matrix that can be used for all three methods. SSCP analyses were performed using the prototype agarose gel matrix for wild-type, polymorphic, and mutant samples from c-Kras exon 12, p53 exons 8 and 9, and HOX2B. We performed SSCP analyses using both isotopic and nonisotopic methods. We also analyzed the samples by deliberate HTX formation and subsequent gel analysis. Using the prototype agarose matrix, we detected single and multiple DNA sequence variants in 150-350 bp fragments with an efficiency comparable to polyacrylamide gels run under similar conditions. For SSCP and HTX assays, we achieved optimal resolution in gels run in vertical formats. However, some HTX samples could be resolved in horizontal gel systems. In addition, based on our studies, we have developed a useful battery of controls and standards for quality control of SSCP and HTX assays. We analyzed several different DNA/protein complexes (SP1, AP2, and octamer binding protein) using the prototype agarose matrix. We obtained good resolution in both vertical and horizontal gel formats. The horizontal gel system is generally superior for this application, due to its ease of use and slightly better resolution.

This new prototype gel matrix offers an alternative for researchers performing analyses that previously could only be done on polyacrylamide type gel matrices. For some applications, this new matrix offers the ease of horizontal gel casting. For all applications, this matrix offers the safety of a nontoxic system and the reproducibility of a thermally gelling system.

2101

Analysis of GLRA1 in familial and sporadic hyperekplexia.
FV Elmslie[1], M Rees[1], A Covanis[2], P Baxter[3], D Gardner-Medwin[3], A Curtis[4], J Burn[4], RM Gardiner[1].
1. Department of Paediatrics, UCL Medical School, London, UK. 2. Aghia Sofia Hospital, Athens, Greece. 3. Newcastle District Hospital, UK. 4. Department of Human Genetics, University of Newcastle, UK.

Hyperekplexia or familial startle syndrome is an autosomal dominant condition, causing an exaggerated startle response to unexpected stimuli. Infants have variable hypertonia in the neonatal period, with a startle reponse causing sustained muscular contraction. The gene was localised to chromosome 5q in 1992 (1) and subsequently mutations were detected in the alpha$_1$ subunit of the glycine receptor in four large pedigrees with hyperekplexia (2).

Eight probands with hyperekplexia have been ascertained, including three sporadic and four familial cases. In one pedigree hyperekplexia appears to be associated with spastic paraparesis, and the hyperekplexia/spastic paraparesis is linked to marker loci on chromosome 5q (3).

Genomic DNA was extracted from white cells. GLRA1 exons were amplified from genomic DNA by PCR using specific oligonucleotide primers (courtesy of Dr J Wasmuth). Amplified exon 6 fragments are being screened for the previously described mutations by restriction enzyme analysis using BfaI and XhoI. In addition, all exons have been screened by single-stranded conformational polymorphism (SSCP) and are currently being screened by heteroduplex analysis. No sequence changes have been detected to date. Products displaying aberrant bands on screening will be analysed further by di-deoxy sequencing.

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2102

Progress in Gene Targeting and Gene Therapy for Retinitis Pigmentosa. (G.J.Farrar, M M.Humphries, A.Erven, P.Kenna,D.Sheils, P.Creighton, R.McCarrick,F.C.Mansergh and P.Humphries). Wellcome Ocular Genetics Unit, Trinity College, Dublin 2, Ireland.

Previously we localised disease genes involved in Retinitis Pigmentosa (RP), an inherited retinal degeneration, close to the rhodopsin and peripherin genes on 3q and 6p. Subsequently, we and others identified mutations in these genes in RP patients. Knowledge of the pathogenesis of some forms of RP has stimulated the establishment of methods to elucidate mechanisms by which mutant proteins result in photoreceptor cell death. Currently animal models for human retinopathies are being generated using gene targeting by homologous recombination in embryonic stem (ES) cells. Genomic clones for retinal genes including rhodopsin and peripherin have been obtained from a phage library carrying mouse DNA isogenic with the ES cell line (CC1.2). The peripherin clone has been sequenced to establish the genomic structure of the mouse gene. Targeting vectors for rhodopsin and peripherin including a neomycin cassette for positive selection and thymidine kinase genes enabling selection against random intergrants are under construction. Progress in vector construction will be presented.

Simultaneously we are developing systems for delivery of gene therapies to retinal tissues utilising replication deficient adenovirus (Ad5). Efficacy of infection subsequent to various methods of intraocular injection and with varying viral titers is being assayed using an adenovirus construct containing a CMV promoter LacZ fusion as reporter and the range of tissues infected and the level and duration of LacZ expression monitored. Retinas from transgenic mice (Rho-31) containing a rhodopsin promoter LacZ fusion directing expression to photoreceptor cells are being used as positive controls for histological procedures. Data on possible viral infection of optic nerve and brain tissues after injection will also be reported. Viral constructs with the LacZ reporter gene under the control of retinal specific promoters such as rhodopsin and IRBP cloned into pXCJL.1 are under construction. An update on developments in photoreceptor cell directed expression of virally delivered genes will be presented. (This research is supported by the Wellcome Trust, the US National, British and Irish RP Societies, the Gund Foundation, the EU Science program and the Ulverscroft Foundation).

2103

Correction of fumarylacetoacetate hydrolase deficiency (type I tyrosinemia) in cultured human fibroblasts by retroviral-mediated gene transfer. ((D. Phaneuf[1], M. Hadchouel[2], C. Bréchot[1], N. Ferry[1] and R.M. Tanguay[3].)) (1) INSERM U370, CHU Necker Enfants Malades, Paris, France (2) INSERM U347, CHU Kremlin Bicêtre, France and (3) Laboratoire de génétique cellulaire et développementale, Université Laval, Ste-Foy (Québec), Canada.

Type I hereditary tyrosinemia (HTI) results from an inherited deficiency in fumarylacetoacetate hydrolase (FAH), the enzyme involved in the last step in tyrosine catabolic pathway. We have constructed recombinant retroviral vectors carrying the cDNA encoding human FAH. In the present report we show that these vectors are able to restore FAH activity stably in primary fibroblasts from HTI patients. Moreover, infected fibroblats displayed an increased resistance to the toxic effect of fumarylacetoacetate, the substrate of FAH which accumulates in HTI. The possibility to express FAH stably in deficient patients represents a first step towards future gene therapy for type I hereditary tyrosinemia.

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Functional Abnormalities in Transgenic Mice Expressing a Mutant Rhodopsin Gene

Yoshinobu Goto,† Neal S. Peachey,*† Harris Ripps,‡ and Muna I. Naash‡§*

Purpose. To evaluate the consequences of the expression of a mutant mouse opsin gene on rod- and cone-mediated function. Experimental conditions were chosen to provide a basis of comparison to the results reported for patients with autosomal dominant retinitis pigmentosa (ADRP) in whom the proline at position 23 has been replaced by a histidine (P23H).

Methods. The mutated gene product resulted in three substitutions in the rhodopsin molecule: P23H, glycine for valine at position 20 (V20G), and leucine for proline at position 27 (P27L). Mice positive for the transgene were differentiated from normal littermates by the polymerase chain reaction. Electroretinograms (ERGs) were obtained from anesthetized mice between 1 and 9 months of age. After photically bleaching approximately 18% of the available rhodopsin, the time course of rod dark adaptation was examined by monitoring rod ERG amplitude recovery. Rhodopsin densitometry was used to determine the relative amounts of rhodopsin in the retinae of normal and transgenic mice.

Results. ERGs obtained from transgenic mice showed a significant reduction in rod-mediated response amplitude at 1 month of age and a relatively slow progressive decrease thereafter. Cone-mediated ERGs, on the other hand, were nearly normal in amplitude for approximately the first 5 months after birth, but at later ages response amplitudes also underwent a progressive decline. In the normal retina, rod ERG amplitudes returned to prebleach levels within 30 minutes, whereas in transgenic mice response amplitudes did not recover within a 2-hour test period. The age-related decline in rod-mediated electroretinal potentials seen in transgenic mice was paralleled by a concomitant fall in rhodopsin density, and the sensitivity losses obtained electroretinographically could be accounted for solely on the basis of reduced quantal absorption.

Conclusions. The pattern of functional changes seen in the transgenic mice are in good agreement with those reported in patients with ADRP with the P23H mutation in the rhodopsin gene. Particularly noteworthy is the fact that the changes in rhodopsin density and visual sensitivity are associated with a progressive shortening of the rod outer segments; the histologic changes induced by the disease process in patients with ADRP have yet to be determined. Invest Ophthalmol Vis Sci. 1995;36:62–71.

Genomic analysis of patients with autosomal dominant retinitis pigmentosa (ADRP) has provided convincing evidence that various subtypes of ADRP can result from mutations in the gene encoding rod opsin, a protein that binds 11-*cis*-retinal to form the visual pigment, rhodopsin.[1–8] The genetic defect results in point mutations in one or another of the amino acids that constitute the polypeptide chain in the disk membranes of the rod outer segments and leads ultimately to the loss of visual cells and blindness.

Transgenic mice with rhodopsin mutations at comparable sites exhibit disorders of a similar nature.[9–11] Recently, Naash et al[10,11] described a slow retinal degeneration in transgenic mice expressing an opsin gene with three point mutations within a seven amino acid sequence near the N-terminus. One of these mutations involves the replacement of proline with histidine at position 23 (P23H), a substitution that occurs also in human patients with ADRP.[1–3,15] The other two mutations, substituting glycine for valine at position 20 (V20G) and leucine for proline at position 27 (P27L), have not been associated with

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Supported by the Department of Veterans Affairs, Washington, DC, by grants from the National Retinitis Pigmentosa Foundation, Baltimore, Maryland; the National Eye Institute (EY10609, EY06516), Bethesda Maryland; the Illinois Society for the Prevention of Blindness, Chicago; the Illinois Eye Fund, Chicago; Midwest Eye Banks and Transplantation Center, Ann Arbor, Michigan; and the Knights Templar Eye Foundation, Springfield, Illinois; and by an unrestricted award from Research to Prevent Blindness, New York, New York.

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Proprietary interest category: N.

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human ADRP, and they were included to enhance antibody recognition of the mutant rhodopsin and differentiation of normal and transgenic mice. Animals expressing the mutated opsin gene will be referred to as VPP mice. These mice display retinal abnormalities that mimic in two important respects the features of human ADRP in which the P23H mutation has been implicated: (i) the degenerative changes involve both rod and cone photoreceptors, with a concomitant decline in the magnitude of the light-evoked electroretinogram (ERG), and (ii) the visual loss progresses at a comparatively slow rate; visual function, measured electroretinographically, is retained for several months, a relatively long period in the life of animals whose longevity rarely exceeds 2 years. In addition to these functional aberrations, earlier studies[10,11] have demonstrated an age-dependent reduction in the length of the rod outer segments and a decrease in the cellular content of the outer nuclear layer.

In the present study, we provide additional evidence that the transgenic mice mimic the human condition in terms of the relative losses of rod- and cone-mediated function and the time course of recovery of rod-mediated function after exposure to a bright, light-adapting field. In addition, we have attempted to address more directly the putative mechanisms that link the gene mutation with the pathogenesis of the degenerative process. One interesting hypothesis, put forth recently[16] to account for the progressive loss of visual cells and the decline in photic sensitivity, suggests that the mutation in some forms of retinitis pigmentosa (RP) may lead to the formation of a 'constitutively active' rhodopsin,[17,18] that is, a molecular species that continuously activates the transduction cascade and presumably induces pathologic changes that mimic the toxic effects of prolonged light exposure. To examine this possibility in VPP mice, we analyzed the electroretinographic responses and rhodopsin content of transgenic animals relative to that of normal littermates. Our results do not provide support for the notion that the opsin mutation leads to a constitutively active rhodopsin in this transgenic model of ADRP. Rather, sensitivity losses in the dark-adapted retina can be attributed to reduced quantal absorption by rhodopsin, a consequence of the reduction in the length of the rod outer segments and the concomitant loss of visual cells.[10,11,19]

MATERIALS AND METHODS

Transgenic Mice

As described previously,[10,11] the transgene was constructed by oligonucleotide-directed mutagenesis and insertion of a mutated fragment into bovine 3OI comprising the complete murine opsin gene. The final transgene consisted of a 15-kb mouse opsin genomic fragment that contained 6-kb upstream and 3.5-kb downstream sequences, and in which a part of the wild type exon 1 of the opsin gene was replaced by a fragment containing the mutations. Transgenic mice were identified by the presence of the restriction fragment length polymorphism that was created in the transgene. Polymerase chain reaction amplification of exon 1 with primers W75/W11, followed by digestion with NcoI, reveals three fragments in wild-type mice of 689 bp, 431 bp, and 197 bp. Transgenic mice have an additional (689 bp + 197 bp) fragment due to the deletion of the NcoI site. The retinal degeneration is inherited as a dominant trait in a Mendelian fashion. Therefore, in the heterozygote to normal matings used to generate the mice studied here, ~50% of the offspring are transgenic and ~50% are normal. Each mouse was tested without the experimenter knowing if the mouse was transgenic or normal. All procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Electroretinography

Mice were anesthetized with an intraperitoneal injection of 15 μl/g body weight of a saline solution containing ketamine (1 mg/ml), xylazine (0.4 mg/ml), and urethane (40 mg/ml). The pupil was dilated with 2.5% phenylephrine HCl, and the animals were placed on a heating pad. Electroretinograms were recorded using a coiled stainless steel wire contacting the anesthetized (1% proparacaine HCl) corneal surface through a layer of 1% methylcellulose. A similar wire placed in the mouth and a needle electrode inserted in the tail served as reference and ground leads, respectively. Responses were differentially amplified (half bandpass: 1 to 1000 Hz), averaged, and stored using a Nicolet (Madison, WI) Pathfinder II signal averaging system. Strobe flash stimuli ($t < 1$ msec) were presented in a Nicolet ganzfeld (GS-2000), either in the dark or superimposed on an adapting field.

Electroretinograms were recorded in two sessions. In the first, dark-adapted, luminance-response functions were obtained using flash luminances ranging from −3.13 to 0.85 log cd-sec/m^2. Stimuli were presented in order of increasing luminance, and the responses to two successive flashes at each intensity were averaged. A 30-second interflash interval was used for the five lowest luminance flashes; a 1-minute interval was used for the eight higher luminances. The animals were then exposed to a 1.60 log cd/m^2 light-adapting field, and, after a period of 10 minutes during which responses reached a stable amplitude,[20] ERGs were elicited at flash luminances ranging from −0.23 to 0.85 log cd-sec/m^2. At each luminance, responses to 50 successive flashes presented at a rate of 2.3 Hz were averaged.

In the second session, ERG recordings were ob-

tained during dark adaptation after a 3-minute exposure to a bright preadapting field (2.4 log cd/m^2) that bleached approximately 18% of the rhodopsin content of the retina. This study was limited to 2-month-old mice, because in older transgenic animals the ERGs were too small to record during the initial phase of dark adaptation. For each animal, five baseline (dark-adapted) responses were recorded to a standard low luminance flash (−2.12 log cd-sec/m^2). After the light-adapting field was extinguished, the standard flash was used to monitor response recovery at various times in darkness. This protocol was continued until ERG amplitude returned to baseline or the mouse awoke from anesthesia. In several mice, a control condition was used to determine the contribution of cone-mediated activity to the dark-adapted ERG. For these experiments, stimuli were superimposed on an adapting field (0.91 log cd/m^2) that suppressed rod function but had little effect on cone-mediated potentials.[21]

Rhodopsin Densitometry

In situ measurements of rhodopsin density were obtained with a modified microscope-based fundus reflectometer[22] adapted for measurements on isolated retinas mounted receptor-side up on a circular disk of filter paper. Details of the rapid scan, computer-based instrument have been described previously.[23] Briefly, absorbance difference spectra were derived from transmissivity data recorded at 25 wavelengths ranging from 410 nm to 700 nm. The density differences [ΔD_λ] represent the wavelength variation in retinal transmissivity between scans recorded from a dark-adapted retina and again after the retina had been exposed for 2 minutes to an intense yellow light (Wratten 16; 460 mW/mm^2) that bleached virtually the full complement of available rhodopsin in the test area of the measuring beam. It is important to note that the value of ΔD_λ does not represent the rhodopsin density for light passing axially through the rod outer segments. Even when measured in vivo, the absorbance changes are diluted by stray light, that is, that fraction of the light reaching the photocell that has passed through the interstices between photoreceptors.[24] In the isolated retina, additional factors contribute to significant reductions in the measured absorbance changes, namely, disorientation of receptor outer segments with respect to the path of the incident light, and the loss of outer segments during the detachment and removal of the retina.[25] Nevertheless, the technique provides a reliable means by which to compare the relative content of rhodopsin in normal and diseased retinae.[15,19,26–28]

RESULTS

Figure 1 shows representative ERG recordings from a 2-month-old normal mouse and from VPP mice of various ages in response to flash stimuli delivered under dark-adapted conditions (Fig. 1A) and presented on a steady background field of 1.60 log cd/m^2 (Fig. 1B). Flash luminance was the maximum available (0.85 log cd-sec/m^2). It is evident that the amplitudes of the dark-adapted responses from transgenic animals were reduced as early as 1 month after birth and that there was a further reduction in the recordings from older animals. The light-adapted recordings from VPP mice gave a somewhat different picture (Fig. 1B). Response amplitudes appeared to be relatively unaffected until approximately 5 months of age and declined thereafter; by 9 months of age, however, the light-adapted response also was markedly reduced.

Rod and Cone Contributions to the ERG Waveform

There is good evidence to indicate that mouse ERGs evoked by incremental flashes superimposed on a background luminance of 1.60 log cd/m^2 (Fig. 1B) are mediated by the cone mechanism,[20,29] but the relative contributions of the rod and cone systems to the dark-adapted potentials are not immediately apparent. Figure 2 shows results that address this question experimentally. In Figure 2A, the test flash was delivered first to the dark-adapted eye (dashed lines) and then on a background field (0.91 log cd/m^2, continuous lines) that exerted a strong suppressive effect on rod-mediated responses but had little effect on cone-mediated potentials.[21] The recordings from a normal (N) and a 3-month-old transgenic (VPP) mouse indicate that the cone-mediated receptor potential makes only a negligible contribution to the dark-adapted a-wave in normal and transgenic mice. However, there is a substantial cone contribution to the dark-adapted b-wave recorded from the normal retina, and this component becomes even more prominent in the dark-adapted responses of the VPP mice owing to the profound loss of rod-mediated activity. On the other hand, when a dim test flash was presented to the dark-adapted eye, a b-wave-dominated response was elicited (Fig. 2B, dashed line); in this case, the test flash was reduced by almost 3 log units (to −2.12 log cd-sec/m^2) as compared with that of Figure 2A. More important, no response was detectable to the same test flash when superimposed on the weak adapting field (Fig. 2B, solid line), indicating that only rod-mediated responses are activated at this stimulus intensity.

Progressive Changes in the Electroretinal Potentials

The age-related changes in dark- and light-adapted ERG components of transgenic mice are illustrated in Figures 2C and 2D, respectively. As mentioned earlier, a significant reduction in the ERG amplitude was already evident in the dark-adapted responses of VPP mice at 1 month of age (Fig. 2C). Both the a- and b-

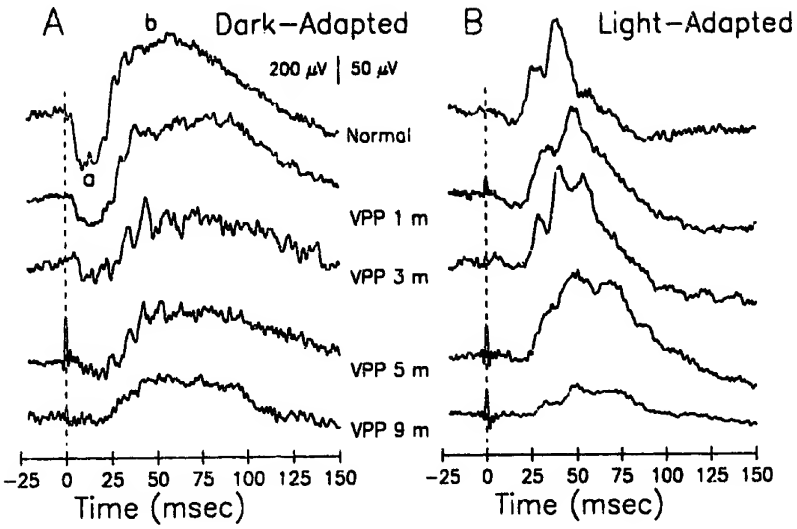

FIGURE 1. Electroretinograms obtained from representative normal and VPP mice of different ages to a 0.85 log cd-sec/m^2 flash stimulus (**A**) presented to the dark-adapted eye, or (**B**) superimposed on a background field of 1.60 log cd/m^2. Vertical dashed lines indicate stimulus onset. The amplitude of the a-wave was measured from the prestimulus baseline to the trough of the cornea-negative peak (a). The b-wave was measured from the a-wave trough to the positive peak (b); b-wave implicit times (see Fig. 4) represent the interval from flash onset to the b-wave peak.

wave potentials were approximately one half of normal at this stage and declined progressively in the responses from older animals. By 9 months of age, the a-wave was no longer detectable with the recording methods used here, but the b-wave was still in evidence. The apparent retention of the dark-adapted b-wave probably reflects the greater photic sensitivity of this response component[30] as well as an underlying contribution from the cone-mediated b-wave potential (Fig. 2A).

Responses recorded under light-adapted conditions are derived almost exclusively from cone-mediated activity and, as shown in Figure 2D, exhibited very different age-related defects. The b-wave responses were only minimally reduced for the first 5 months after birth, and, although there was a gradual decline thereafter, response amplitudes recorded from 9-month-old animals were still about one third of normal at this relatively advanced age.

The Temporal Course of Dark Adaptation

Figure 3 illustrates the time-dependent changes in the ERG b-wave after exposure to a preadapting light (2.4 log cd/m^2) that bleaches approximately 18% of the available rhodopsin in a normal retina. All responses were elicited with a constant test luminance (−2.12 log cd-sec/m^2); note that with this intensity test flash (see Fig. 2B), only the rod system was activated throughout the course of dark adaptation. To minimize intersubject differences in absolute amplitude, responses for each mouse are expressed as a percentage of the average dark-adapted baseline for that mouse. After extinguishing the light-adapting field, normal mice (open circles) required approximately 10 minutes for amplitude to recover one half its original magnitude and approximately 25 to 30 minutes in darkness to return to the initial dark-adapted level. In normal mice, the response amplitudes overshot the original baseline values. This could be a property of the normal retina or may indicate that the retina had not been fully dark adapted at the onset of light adaptation. Data from the VPP mice (filled circles) show that the recovery of response amplitude during dark adaptation was grossly abnormal. The rate of recovery was retarded ($t_{1/2}$ = 40 minutes), and response amplitudes did not reach the preexposure level within a 2-hour period of dark adaptation.

Rhodopsin and Retinal Sensitivity

Table 1 presents data on the rhodopsin density measurements and a-wave amplitude recordings from normal animals and VPP mice at 1 and 2 months of age. Rhodopsin values represent absorbance measurements (ΔD_λ) at the peak of the difference spectrum (λ = 510 nm; see Fig. 4B). Note that at comparable ages the two metrics are reduced relative to the normal by approximately the same proportion. Thus, at 1 month of age, the values for ΔD_{510} and for the a-wave amplitude are reduced to 45% and 46% of normal, respectively, whereas at 2 months, the corresponding

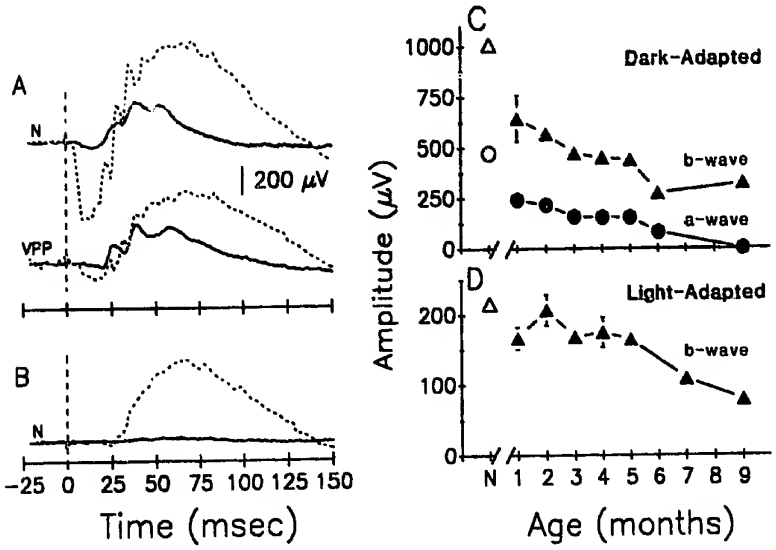

FIGURE 2. (A) Electroretinographic recordings from a normal (N) and a VPP mouse to a 0.85 log cd-sec/m^2 flash stimulus presented in the dark (*dotted lines*) or against a 0.91 log cd/m^2 adapting field (*continuous lines*). Vertical dashed lines indicate the time of flash presentation. (B) Responses from a normal mouse elicited with the dim flash used to monitor dark adaptation (−2.12 log cd-sec/m^2) presented under the same adaptation conditions as in (A). Note the absence of an underlying cone component in both the dark- and light-adapted responses. (C) Age-related changes in the amplitude of the dark-adapted ERG a-wave (*circles*) and b-wave (*triangles*). Open symbols represent the mean of 28 normal mice. Seven to 10 VPP mice were tested at 1 to 4 months; three to four VPP mice were tested at later ages. Error bars indicate ±1 SEM and are omitted when smaller than the plotted point. (D) Age-related changes in the amplitude of the cone electroretinographic b-wave; symbols as in (C).

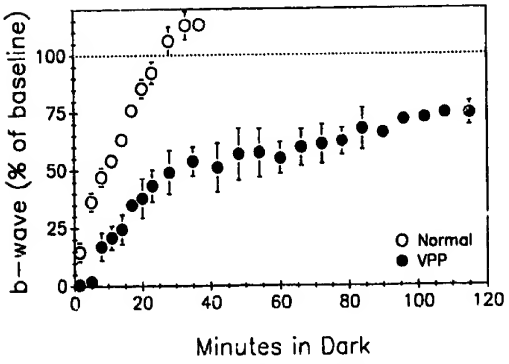

FIGURE 3. Rod b-wave amplitude recovery during dark adaptation after the termination of a 2.4 log cd/m^2 background field; all responses were elicited with a constant flash luminance of −2.12 log cd-sec/m^2. Amplitudes obtained from each mouse are normalized to the average baseline response obtained after dark adaptation. Open circles represent averaged (±1 SEM) results obtained from six normal 2-month-old mice. Filled circles indicate results obtained from four 2-month-old VPP mice.

reductions are to 32% and 25% of normal. As shown in Figure 4A, this relation applies also when retinal thresholds, derived from ERG measurements, are plotted as a function of the rhodopsin density, expressed as percent of normal. Three different indices of threshold were used: (i) the luminance required to generate a criterion a-wave response of 50 μV (ii) the luminance giving rise to a 100 μV b-wave, and (iii) the luminance required to evoke a b-wave with an implicit time of 80 msec (a point that falls on the linear portion of the function relating b-wave implicit time to stimulus luminance; see Fig. 4C). The data for each of these threshold measures is approximated by a curve (solid line) that relates threshold elevations to a decrease in the probability of quantal absorption by rhodopsin.[22] This so-called "probability function" has been shown to hold in patients with various forms of RP[15,26,27] and for conditions in which the rhodopsin density measurements are low due to a reduced length of the rod outer segments, for example, in the developing retina.[31] The dashed line shows results obtained in rat,[32] and represents the log–linear relation between b-wave threshold and rhodopsin density that applies to situations in which a fraction of the native rhodopsin is

TABLE 1. Rhodopsin Density and Maximum a-Wave Amplitudes of Normal and Transgenic Mice

	Number of Animals	*Rhodopsin ΔD_{510}*	*ERG a-wave (μV)*
Normal	7	0.152 ± 0.011	523.5 ± 57.5
VPP 1 month	6	0.070 ± 0.005	233.5 ± 10.0
VPP 2 month	6	0.048 ± 0.008	131.4 ± 18.1

ERGs were recorded from mice that were dark adapted overnight; a-wave data are for responses to a 0.65 log cd·sec/m^2 flash stimulus. One to 2 days later, the same mice were again dark adapted and killed, and the retinas were isolated for rhodopsin densitometry. Tabulated values give the mean ± SEM.

depleted either by bleaching[32] or as a consequence of vitamin A deficiency.[26,33–36] It is clear that the data obtained from the VPP mice are not fit well by the log–linear relation.

DISCUSSION

The main objectives of the present study were to examine the effects on retinal function of the expression of the VPP point mutations in the murine rhodopsin gene and to assess some of the factors that might be responsible for the genetically induced abnormalities in structure and function. These mutations result in a slow degeneration of the rod photoreceptors, as demonstrated both histologically[10,11] and by the gradual reduction in a-wave amplitude of the rod-mediated ERG (Figs. 1A, 2C). Moreover, it appears that the VPP mutations exert an effect on the development of the rod photoreceptors. Although the inner retina develops normally and the outer nuclear layer appears initially to contain a normal complement of photoreceptor nuclei, the rod outer segments never achieve the length seen in age-matched normal mice and they become progressively shorter as the disease progresses.[10,11] This is consistent with the observation that the rod receptor potential (a-wave) is reduced by ~50% at the earliest age (1 month) at which the ERG was tested (Fig. 1A) and declines as a function of age (Fig. 2C). Because the transgene is expressed only in rods, the VPP mutations would be expected to spare initially the cone system, and the electroretinographic results indicate that this is indeed the case. The cone-mediated ERG amplitudes remained relatively normal until approximately 5 months of age, and only then did they begin to decrease (Figs. 1B, 2D). This observation suggests that cone dysfunction occurs subsequent to rod degeneration in VPP mice and reflects perhaps a structural disorganization of cone photoreceptors due to the loss of support provided by neighboring rods, or to the effects of some toxic by-product of rod degeneration. In any case, the factors underlying the various anomalies in the cone ERG remain to be identified.

Abnormalities in the recovery of ERG amplitude during dark adaptation (Fig. 3) provide a potentially important insight into the nature of the disease process. It is not possible with noninvasive techniques to identify the molecular basis for the remarkably slow time course over which dark adaptation proceeds in the VPP mice. Nevertheless, it is noteworthy that similar aberrations have been reported for psychophysical measures of dark adaptation in patients who have ADRP with the P23H rhodopsin mutation,[15] as well as in some individuals with RP involving a different rhodopsin mutation [37] or in whom genetic tests were not performed.[38] There are obviously a number of possible explanations to account for this type of anomaly. The delay could reflect a defect in the processes underlying the regeneration of rhodopsin. For example, the VPP mutations might interfere with the process by which rhodopsin is reformed after bleaching. The binding site for the chromophore is located within the transmembrane domain of the opsin molecule,[39] but there is evidence to suggest that the protein–chromophore interaction may be modified by changes in the intradiskal regions of the molecule,[40] including the N-terminus.[41] Thus, there is the possibility that the VPP mutations interfere with the mechanism by which 11-*cis*-retinaldehyde enters the binding pocket of the mutant opsin. Alternatively, a delay in dark adaptation could be caused by prolonged lifetimes of phototransduction byproducts that desensitize the rods.[42] Photoactivated rhodopsin (R^*) is normally inactivated by a sequence of events involving phosphorylation and arrestin binding.[43] Any interference with these interactions by the mutated opsin molecules might produce delays in the recovery of visual sensitivity. However, each of the forgoing hypothetical scenarios implicates processes initiated by the photoactivation of rhodopsin, and there is reason to doubt that these types of abnormalities are instrumental in triggering the disease process. The results of recent studies (Naash et al, manuscript in preparation) have shown that VPP mice reared in darkness from birth undergo a progressive, albeit slower, retinal degeneration similar to that seen in animals reared in cyclic lighting. Determining the mechanism underlying the delayed dark adaptation observed in VPP mice and in patients with the P23H form of ADRP is clearly a significant question for future studies.

There remains, of course, the possibility that the mutant rhodopsin of VPP mice is a constitutively active molecule that gives rise to a persistent 'dark light.' As mentioned earlier, it has been suggested[16] that sustained activation of the transduction process may induce degenerative changes similar to those produced by prolonged exposure to light itself.[44–46] Interestingly, there are a number of point mutations that re-

sult in opsins capable of constitutively activating transducin when xpressed in an in vitro assay system.[17,18,47] However, the presence of thes mutations in humans results in widely disparate disorders, ranging from congenital stationary night blindness[47,48] to a rapidly progressive form of RP.[6] Neither of these conditions is similar in their functional consequences to those expressed by the VPP mice or patients who have ADRP with the P23H mutation in rhodopsin, nor has it been shown unequivocally that under light conditions that lead to photic injury, the activation of transducin per se is the event that induces the degenerative process.

Also inconsistent with the equivalent light hypothesis are the results shown in Figure 4, wherein log thresholds (the logarithm of the luminance required to achieve a criterion response) are plotted as a function of the rhodopsin content of the retina (expressed on a linear scale). Studies on a variety of vertebrate species, including humans, have shown that denuding opsin of its chromophore, whether by photic bleaching[22,32,49] or vitamin A deprivation,[26,34-36] leads to a disproportionately large loss of visual sensitivity relative to that expected simply from the decrease in quantal absorption. For reasons that are still unclear, the results seem to be best approximated by a log-linear relationship; b-wave data from rat[32] provide a good example of this sort of relation (dashed line, Fig. 4A), although the slope of the function may differ for other species.[22] The results obtained for the VPP mice fail to fit a pattern consistent with the equivalent light hypothesis. The data points of Figure 4 do not deviate significantly from the continuous curve that describes the function to be expected if the probability of quantal absorption were the sole determinant of the rise in threshold. In these circumstances, reducing the rhodopsin content of the rods by 50% would produce a twofold (0.3 log unit) rise in threshold, whereas a reduction of 90% would elevate threshold by only tenfold (1 log unit). This is consistent with the short rod outer segments of VPP mice seen histologically

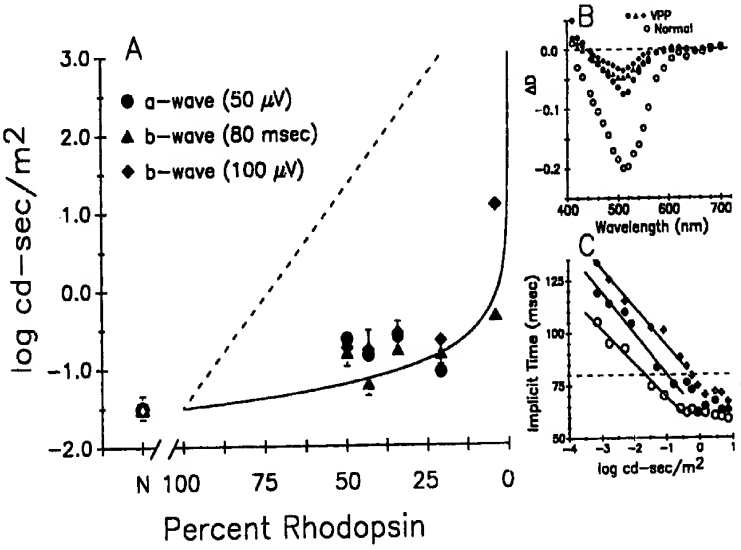

FIGURE 4. (A) The relation between electrically recorded thresholds (1/sensitivity) and the relative content of rhodopsin in normal (*open symbols*) and transgenic (*filled symbols*) mice. Three threshold criteria were used: the intensity required to elicit a 50-μV a-wave (*circles*), a 100-μV b-wave (*diamonds*), or an 80-msec b-wave (*triangles*). Data points for normal mice (*open symbols*) indicate the mean (±1 SEM; $N = 7$); data points for VPP mice represent the averaged (±1 SEM) value for four mice with similar rhodopsin densities, or the value obtained from a 7.5-month-old mouse with a low rhodopsin density (data at far right). Values for the three measures have been adjusted vertically in order that the normal means coincide. The solid line is the function expected if threshold elevation is due solely to a reduction in quantal absorption. The dashed line represents changes in b-wave threshold recorded during the regeneration of bleached rhodopsin in the rat retina.[32] (B) Examples of the absorbance difference spectra (DD_1) obtained from a normal mouse (*open circles*) and from VPP mice at different ages (1 month, *filled circles*; 2 months, *filled triangles*; 3 months, *filled diamonds*). (C) Examples of b-wave implicit time functions. The mean normal function is represented by open circles; individual VPP mice are shown as filled symbols. The solid lines represent the least-squares fit linear regression to the data spanning the linear range of each function. The dashed line indicates the 80-msec criterion from which the threshold luminance was determined.

and with the ERG evidence that these photoreceptors are capable of functioning surprisingly well with this degree of structural impairment.[10]

Although it seems difficult to reconcile the retention of rod-mediated electrical activity with such gross losses in the rhodopsin content of the visual cells, it should be noted that similar findings have been reported in individuals with the P23H rhodopsin mutation,[15] as well as in other patients with ADRP in whom the gene mutations were not identified.[19,26,27] This situation, and the observation that the rod outer segments of VPP mice become progressively shorter with age,[10] raise the possibility that one consequence of the opsin mutation is an imbalance between the rates of disk formation and disk shedding.[19,26] The mutant opsin may induce a more rapid rate of disk shedding, or it may somehow impede the processes by which newly formed opsin is transported to, or inserted into, the disk membranes. In either event, the delicate balance between new disk formation and disk shedding is upset, and there follows a progressive shortening and eventual death of the visual cells.

It is important to recall that the rhodopsin expressed in the VPP mice includes three point mutations: V20G, P23H, and P27L. Although P23H is responsible for some forms of human ADRP,[1-3,15] neither V20G nor P27L have been identified to date in patients with ADRP.[50] This observation, and the fact that proline 23 is highly conserved among normal opsins in a wide variety of species,[51] make it highly likely that the P23H mutation is primarily responsible for the retinal degeneration seen in the VPP mice. Nevertheless, it would be circumspect to determine whether mutations V20G, P27L, or both, contribute in some way to the degenerative process; lines expressing only one or the other of these mutations are necessary to address this possibility. Another fundamental issue that will be important for our understanding of the morphologic and physiological abnormalities observed in the transgenic animals concerns the functional status of the opsin induced by the VPP mutation. At present, we have no evidence as to whether the mutant opsin expressed in the VPP mice is capable of binding retinoid and interacting with the enzymes of the transduction cascade, or indeed whether it is inserted into the rod disk membranes.[52,53] These questions are currently under investigation, and the outcome will obviously influence the interpretation of the data presented in this study.

These issues aside, the results of our experiments have identified several important functional similarities between the VPP mice and human subjects with the P23H form of ADRP. Individuals with the P23H mutation exhibit a relatively slow rod degeneration with initial sparing of cone function,[1,2] an unusually slow rate of dark-adaptation,[15] and threshold elevations that can be ascribed solely to the reduced probability of quantal absorption resulting from the diminished rhodopsin content of the rods.[15] The results of the present study indicate that each of these characteristics is shared by the VPP mice. In view of the decades-long time course of human ADRP, the VPP mice appear to provide a useful animal model in which to examine the mechanisms by which mutations in the N-terminus region of rhodopsin disrupt the functional and structural integrity of the visual cells and lead ultimately to a widespread retinal degeneration.

Key Words

retinitis pigmentosa, electroretinogram, visual sensitivity, rhodopsin density, transgenic mice

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(74) **Mandataire** :

(54) Virus recombinants et leur utilisation en thérapie génique.

(57) La présente invention concerne l'utilisation de virus recombinants défectifs contenant un gène inséré pour la préparation d'une composition pharmaceutique destinée au traitement des pathologies oculaires.

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VIRUS RECOMBINANTS ET LEUR UTILISATION EN THERAPIE GENIQUE

La présente invention concerne de nouveaux virus recombinants, leur préparation et leur utilisation en thérapie génique, pour le transfert et l'expression de gènes au niveau de l'oeil. Elle concerne également des compositions pharmaceutiques comprenant lesdits virus recombinants. Plus particulièrement, la présente invention concerne des virus recombinants défectifs et leur utilisation pour le traitement de pathologies oculaires.

Le traitement des pathologies oculaires, et notamment des maladies héréditaires constitue un problème non résolu actuellement. Parmi ces pathologies, on peut citer par exemple les rétinites pigmentaires, qui résultent d'altérations génétiques, et pour lesquelles aucun traitement n'est actuellement disponible. Par ailleurs, les pathologies non héréditaires telles que les atteintes post-inflammatoires (dégénérescence rétinienne, etc) ne disposent pas non plus aujourd'hui de traitement adapté. En particulier, si l'on tente d'agir préventivement, notamment au moyen de corticoïdes, on ne dispose actuellement d'aucun moyen satisfaisant pour traiter ces atteintes.

Il est donc important de pouvoir disposer d'outils permettant un traitement spécifique, efficace et localisé des pathologies oculaires. La présente invention apporte une solution avantageuse à ce problème, en démontrant la possibilité de traiter les pathologies oculaires par la thérapie génique.

La thérapie génique consiste à corriger une déficience ou une anormalité (mutation, expression aberrante, etc) par introduction d'une information génétique dans la cellule ou l'organe affecté. Cette information génétique peut être introduite soit in vitro dans une cellule extraite de l'organe, la cellule modifiée étant alors réintroduite dans l'organisme, soit directement in vivo dans le tissu approprié. Dans ce second cas, différentes techniques existent, parmi lesquelles des techniques diverses de transfection impliquant des complexes d'ADN et de DEAE-dextran (Pagano et al., J.Virol. 1 (1967) 891), d'ADN et de protéines nucléaires (Kaneda et al., Science 243 (1989) 375), d'ADN et de lipides (Felgner et al., PNAS 84 (1987) 7413), l'emploi de liposomes (Fraley et al., J.Biol.Chem. 255 (1980) 10431), etc. Plus récemment, l'emploi de virus comme vecteurs pour le transfert de gènes est apparu comme une alternative prometteuse à ces techniques physiques de transfection. A cet égard,

différents virus ont été testés pour leur capacité à infecter certaines populations cellulaires. En particulier, les rétrovirus (RSV, HMS, MMS, etc), le virus HSV, les virus adéno-associés, et les adénovirus.

Toutefois, jusqu'à présent, aucun de ces vecteurs n'a été utilisé ni décrit comme utilisable pour le transfert de gènes au niveau de l'oeil. La présente invention constitue la première démonstration qu'il est possible de traiter les pathologies oculaires par la thérapie génique.

Un premier objet de l'invention réside dans l'utilisation d'un virus recombinant défectif contenant un gène inséré pour la préparation d'une composition pharmaceutique destinée au traitement des pathologies oculaires.

Plus particulièrement, on utilise selon la présente invention des virus recombinants défectifs dérivés de virus capables d'infecter et d'exprimer un gène inséré dans les cellules de l'oeil, sans entraîner des phénomènes cytopathologiques ou d'effets pathogènes. Des virus susceptibles d'être utilisés dans l'invention sont par exemple les adénovirus, les virus adéno-associés ou encore le virus HSV.

La présente invention repose plus particulièrement sur la mise en évidence que les virus de type adénovirus sont capables de transférer et d'exprimer des gènes désirés au niveau de l'oeil. Les exemples présentés plus loin montrent que les adénovirus sont capables, selon le mode d'administration, de transférer efficacement, pour une durée importante et sans effet cytopathologique, des gènes dans l'endothélium cornéen, dans les cellules photoréceptrices, dans les cellules du nerf optique, dans les cellules bipolaires, etc. Par ailleurs, compte tenu de l'accès relativemant aisé aux différents compartiments de l'oeil par la microchirurgie (microinjection), ainsi que de l'existence de barrières naturelles dans cet organe (membrane de Descemet, membrane de Bruch, cristallin, etc) la présente invention permet avantageusement d'effectuer un transfert de gènes très ciblé, en fonction de la pathologie à traiter. Les résultats présentés montrent également que l'expression d'un gène désiré est stable sur une longue période (aucune perte d'activité à 50 jours).

Dans un mode de réalisation préféré, l'invention réside dans l'utilisation d'un adénovirus recombinant défectif contenant un gène inséré pour la préparation d'une composition pharmaceutique destinée au traitement des pathologies oculaires.

Le terme "virus ou adénovirus défectif" désigne un virus incapable de se répliquer de façon autonome dans la cellule cible. Généralement, le génome des virus

défectifs utilisés dans le cadre de la présente invention est donc dépourvu au moins des séquences nécessaires à la réplication dudit virus dans la cellule infectée. Ces régions peuvent être soit éliminées (en tout ou en partie), soit rendues non-fonctionnelles, soit substituées par d'autres séquences et notamment par le gène inséré. Préférentiellement, le virus défectif conserve néanmoins les séquences de son génome qui sont nécessaires à l'encapsidation des particules virales.

S'agissant plus particulièrement des adénovirus, il en existe différents sérotypes, dont la structure et les propriétés varient quelque peu. Néanmoins, ces virus ne sont pas pathogènes pour l'homme, et notamment les sujets non immuno-déprimés. Parmi ces sérotypes, on préfère utiliser dans le cadre de la présente invention les adénovirus de type 2 ou 5 (Ad 2 ou Ad 5). Dans le cas des adénovirus Ad 5, les séquences nécessaires à la réplication sont les régions E1A et E1B.

Des virus recombinants défectifs dérivés de rétrovirus, de virus adéno-associés ou du virus HSV (herpes simplex virus) ont déjà été décrits dans la littérature [Roemer et Friedmann, Eur. J. Biochem. 208 (1992) 211 ; Dobson et al., Neuron 5 (1990) 353 ; Chiocca et al., New Biol. 2 (1990) 739 ; Miyanohara et al., New Biol. 4 (1992) 238; WO91/18088].

Au sens de la présente invention, le terme "gène inséré" désigne toute séquence d'ADN introduite dans le virus recombinant, dont l'expression dans la cellule cible est recherchée.

Il peut s'agir en particulier d'un (ou plusieurs) gène(s) de structure codant pour une (des) protéine(s) ou pour une partie d'une (de) protéine(s). La protéine ou partie de protéine ainsi codée peut être une protéine homologue vis-à-vis de la cellule cible (c'est-à-dire une protéine qui est normalement exprimée dans la cellule cible lorsque celle-ci ne présente aucune pathologie), ou une protéine hétérologue vis-à-vis de ladite cellule. Dans le premier cas, l'expression de la protéine permet par exemple de pallier une expression insuffisante dans la cellule ou l'expression d'une protéine inactive ou faiblement active en raison d'une modification, ou encore de surexprimer ladite protéine. Dans le second cas, la protéine exprimée peut par exemple compléter ou apporter une activité déficiente dans la cellule lui permettant de lutter contre une pathologie.

Parmi les gènes insérés au sens de la présente invention, on peut citer plus particulièrement :

- les gènes impliqués dans des pathologies génétiques oculaires,

- les gènes codant pour des facteurs de croissance, des cytokines ou des neurotrophines : Le role protecteur ou curatif du produit d'expression de ces gènes dans différentes pathologies oculaires a été démontré, et notamment sur la détérioration des cellules photoréceptrices sous l'effet de la lumière (Lavail et al., PNAS 89 (1992) 11249).

- les gènes de facteurs de régulation (facteurs de transcription, facteurs de traduction),

- les gènes codant pour des enzymes,

- les gènes codant pour des protéines ayant des propriétés anticancéreuses, telles que les interférons, les facteurs de nécrose des tumeurs, la protéine p53, etc, ou encore,

- les gènes codant pour des antigènes permettant une vaccination (une protection) locale contre une infection de l'oeil.

A titre d'exemples spécifiques, mais non limitatifs, on peut citer :

. le gène de l'ornithine aminotransférase impliqué dans l'atrophie gyrée (Akaki et al., J. Biol. Chem. 267(18) (1992) 12950),

. le gène de la rhodopsine, impliqué dans une forme de rétinite pigmentaire (Dryja et al., Nature 343 (1990) 364),

. le gène de la périphérine RDS, impliqué dans une forme de rétinite pigmentaire (Farrar et al., Nature 354 (1991) 478),

. le gène de la tyrosinase, impliqué dans l'albinisme oculocutanée type B1 (Giebel et al., Am. J. Hum. Genet. 48 (1991) 1159),

. le gène mitochondrial NDI, impliqué dans la maladie de Leber (Howell et al., Am. J. Hum. Genet. 48 (1991) 935),

. le gène de la sous-unité β de la cGMP phosphodiestérase, qui permet de ralentir la dégénérescence rétinienne (Lem et al., PNAS 89 (1992) 4422),

. le gène de la rab géranylgéranyl transférase, dont la déficience semble liée à une dégénérescence rétinienne lors de choroïdermies (Seabra et al., Science 259 (1993) 377),

. le gène du facteur de croissance des fibroblastes basique (bFGF), capable de retarder la dégénérescence des cellules photoréceptrices observée dans certaines dystrophies rétiniennes héréditaires (Faktorovich et al., Nature 347 (1990) 83),

. le gène de l'interleukine-8, qui permet d'induire une néovascularisation dans la cornée (Strieter et al., Am. J. Pathol. 141(6) (1992) 1279).

Le terme "gène inséré" désigne également des séquences antisens, dont l'expression dans la cellule cible permet de contrôler l'expression de gènes ou la transcription d'ARNm cellulaires. De telles séquences peuvent par exemple être transcrites, dans la cellule cible, en ARN complémentaires d'ARNm cellulaires et bloquer ainsi leur traduction en protéine.

Généralement, le gène inséré comprend également des séquences permettant son expression dans la cellule infectée. Il peut s'agir des séquences qui sont naturellement responsables de l'expression dudit gène lorsque ces séquences sont susceptibles de fonctionner dans la cellule infectée. Il peut également s'agir de séquences d'origine différente (responsables de l'expression d'autres protéines, ou même synthétiques). Notamment, il peut s'agir de séquences issues du génome de la cellule que l'on désire infecter, ou du génome du virus utilisé. S'agissant d'adénovirus, on peut citer par exemple les promoteurs des gènes E1A, MLP, etc. En outre, ces séquences d'expression peuvent être modifiées par addition de séquences d'activation, de régulation, etc. Par ailleurs, lorsque le gène inséré ne comporte pas de séquences d'expression, il peut être inséré dans le génome du virus défectif en aval d'une telle séquence.

Dans ce qui suit sont décrites plus en détail la construction et l'utilisation d'adénovirus recombinants défectifs. Il est entendu néanmoins que cette description peut être appliquée par l'homme du métier aux autres virus suceptibles d'être utilisés dans le cadre de la présente invention, ainsi qu'indiqués plus haut.

Les adénovirus recombinants défectifs peuvent être préparés par recombinaison homologue entre un adénovirus et un plasmide portant entre autre le gène que l'on désire insérer. La recombinaison homologue se produit après co-transfection desdits adénovirus et plasmide dans une lignée cellulaire appropriée. La lignée cellulaire utilisée doit de préférence (i) être transformable par lesdits éléments, et (ii), comporter les séquences capables de complémenter la partie du génome de l'adénovirus défectif, de préférence sous forme intégrée pour éviter les risques de recombinaison. A titre d'exemple de lignée, on peut mentionner la lignée de rein embryonnaire humain 293 (Graham et al., J. Gen. Virol. 36 (1977) 59) qui contient notamment, intégrée dans son génome, la partie gauche du génome d'un adénovirus Ad5 (12 %).

Ensuite, les vecteurs qui se sont multipliés sont récupérés et purifiés selon les techniques classiques de biologie moléculaire.

La présente invention concerne également une composition pharmaceutique comprenant une quantité suffisante de virus recombinant défectif tel que décrit précédemment, sous une forme adaptée à un usage oculaire.

En particulier, le virus recombinant défectif peut être sous forme de solution injectable, de collyre, de pommade ophtalmique, etc. Les véhicules pharmaceutiquement acceptables pour de telles formulations adaptées à un usage oculaire sont notamment des solutions salines (phosphate monosodique, disodique, clrorure de sodium, potassium, calcium ou magnésium, etc, ou des mélanges de tels sels), la vaseline, l'huile de vaseline, etc.

Dans le cas de collyres ou de pommades ophtalmiques, il est entendu que les applications thérapeutiques peuvent être plus limitées en raison d'une diffusion plus faible du virus recombinant défectif.

Dans leur utilisation pour le traitement des pathologies oculaires, les virus recombinants défectifs selon l'invention peuvent être administrés selon différents modes, et notamment par injection sousrétinale, éventuellement précédée d'une vitrectomie, ou par injection intravitreuse, simples ou multiples (voir figure 1). L'injection sous-rétinale peut être réalisée sélectivement dans différents compartiments de l'oeil, et notamment, l'injection peut être réalisée au niveau du vitré, de la chambre anterieure ou de l'espace rétrobulbaire. Les résultats présentés dans la présente demande montrent que ces différents modes d'injection permettent d'infecter de manière ciblée les différents tissus de l'oeil, et notamment, l'endothélium cornéen, les cellules photoréceptrices, les cellules bipolaires, les cellules ganglionaires ou encore les cellules des muscles oculomoteurs.

Les doses de virus utilisées pour l'injection peuvent être adaptées en fonction de différents paramètres, et notamment en fonction du mode d'administration utilisé, de la pathologie concernée, du gène à exprimer, ou encore de la durée du traitement recherchée. D'une manière générale, les adénovirus recombinants selon l'invention sont formulés et administrés sous forme de doses comprises entre 10^4 et 10^{14} pfu/ml, et de préférence 10^6 à 10^{10} pfu/ml. Le terme pfu ("plaque forming unit") correspond au pouvoir infectieux d'une solution de virus, et est déterminé par infection d'une culture cellulaire appropriée, et mesure, généralement après 48 heures, du nombre de plages

de cellules infectées. Les techniques de détermination du titre pfu d'une solution virale sont bien documentées dans la littérature.

Compte tenu de la stabilité d'expression du gène inséré dans la cellule cible, la présente invention devrait permettre de traiter la majorité des pathologies oculaires avec peu d'injections.

La présente invention offre ainsi un moyen très efficace pour le traitement des pathologies oculaires, et notamment celles dont les mécanismes ont été élucidés au niveau moléculaire. En particulier, l'implication de gènes a été démontrée dans l'atrophie gyrée, dans la maladie de Norrie (Hum. Mol. Genet. 1(7) (1992) 461), dans la dégénérescence rétinienne (Bowes et al., PNAS 86 (1989) 9722), dans la maladie de Leber, dans les choroïdermies (Cremers et al., Nature 347 (1990) 674), dans la dégénérescence des cellules photoréceptrices, dans les rétinites pigmentaires, dans l'albinisme, dans le syndrome Kearns-Sayre (Shoffner et al., PNAS 86 (1989) 7952), etc. La présente invention est également pour le traitement des altérations de la cornée acquises résultant de maladies inflammatoires, des atteintes rétiniennes post-inflammatoires, etc.

La présente invention rend également possible la thérapie par les protéines ou peptides, dont l'utilisation par les voies classiques d'administration est très hypothétique en raison de leur forte sensibilité aux mécanismes de dégradation et d'élimination de l'organisme, et des problèmes liés à la pénétration dans les cellules. L'emploi de virus selon l'invention permet d'exprimer directement à l'interieur de la population de cellules ciblées le polypeptide ou la protéine désirée, qui n'est donc plus accessible aux mécanismes mentionnés ci-avant.

L'ensemble des résultats présentés dans la présente demande démontre plus particulièrement que les adénovirus recombinants, défectifs pour la réplication, constituent des vecteurs particulièrement intéressants pour le transfert de gènes in vivo dans les cellules oculaires. Les expériences réalisées montrent la possibilité d'une expression stable à long-terme de gènes dans ces cellules. En particulier, une expression stable est observée 50 jours après l'injection. De plus, le spectre d'expression large dans les différentes cellules oculaires constitue également un résultat particulièrement intéressant dans la mesure où pratiquement toutes les maladies de la rétine (notamment la retinitis pigmentosa) affectent une grande surface de la rétine.

En outre, ce traitement peut concerner aussi bien l'homme que tout animal tel que les ovins, les bovins, les animaux domestiques (chiens, chats, etc), les chevaux, les poissons, etc.

La présente invention est plus complètement décrite à l'aide des exemples qui suivent, qui doivent être considérés comme illustratifs et non limitatifs.

Légende des figures

Figure 1 : Représentation schématique de l'oeil. C = cornée; AC = Chambre antérieure; L = cristallin; V = vitré; I = iris; ON = nerf optique; R = espace rétrobulbaire.

Construction d'un adénovirus recombinant défectif (Ad.RSVβGal) :

La procédure générale permettant la préparation des adénovirus recombinants a été décrite dans la partie générale de la description.

L'adénovirus Ad.RSVβGal est un adénovirus recombinant défectif (délété des régions E1 et E3) obtenu par recombinaison homologue in vivo entre l'adénovirus mutant Ad-d1324 (Thimmappaya et al., Cell 31 (1982) 543) et le plasmide pAd.RSVβGal (Akli et al. 1993).

Le plasmide pAd.RSVβGal contient, dans l'orientation 5'->3',

- le fragment PvuII correspondant à l'extrémité gauche de l'adénovirus Ad5 comprenant : la séquence ITR, l'origine de réplication, les signaux d'encapsidation et l'amplificateur E1A;
- le gène codant pour la β-galactosidase sous le contrôle du promoteur RSV (du virus du sarcome de Rous),
- un second fragment du génome de l'adénovirus Ad5, qui permet la recombinaison homologue entre le plasmide pAd.RSVβGal et l'adénovirus d1324.

Après linéarisation par l'enzyme ClaI, le plasmide pAd.RSVβGal et l'adénovirus d1324 sont co-transfectés dans la lignée 293 en présence de phosphate de calcium pour permettre la recombinaison homologue. Les adénovirus recombinants ainsi générés sont sélectionnés par purification sur plaque. Après isolement, l'ADN de l'adénovirus recombinant est amplifié dans la lignée cellulaire 293, ce qui conduit à un surnageant de culture contenant l'adénovirus défectif recombinant non purifié ayant un titre d'environ 10^{10} pfu/ml.

Les particules virales sont généralement purifiées par centrifugation sur gradient de chlorure de césium selon les techniques connues (voir notamment Graham et al., Virology 52 (1973) 456). L'adénovirus Ad.RSVβGal est conservé à -80°C dans 20 % de glycérol. Avant injection, la suspension d'adénovirus est diluée au tiers dans un tampon phosphate PBS.

Injection in vivo

- Protocole

Des souris C57Bl/6 de 3 à 7 semaines ont été anesthésiées avec de l'avertine. Dans chaque oeil a ensuite été injecté 10^7 à 10^8 pfu d'adénovirus recombinant Ad.RSVβGal, soit au niveau de la chambre anterieure, soit au niveau du vitré, soit au niveau de l'espace rétrobulbaire (voir figure 1). Les animaux ont été sacrifiés 7 à 50 jours après l'injection par dislocation cervicale et les yeux ont été récupérés et fixés dans l'azote liquide. Des sections sagitales et coronales (10-15 µm) sont réalisées sur cryostat, puis colorées en présence de X-gal pour révéler l'activité β-galactosidase qui peut être visualisée par l'apparition d'une coloration bleue dans le noyau des cellules infectées, et contre-colorées avec de l'hémotoxyline et de l'éosine.

- Injection au niveau de la chambre anterieure

Après injection de 10^8 pfu d'adénovirus Ad.RSVβGal au niveau de l'espace de la chambre anterieure, seules les cellules de la couche endotheliale expriment l'activité β-galactosidase. En revanche, les cellules épithéliales ou du stroma ne présentent aucune coloration à la suite d'une telle injection. De plus, les cellules marquées (infectées) sont distribuées régulièrement dans la couche endothéliale, quel que soit le temps d'administration. Ce résultat montre que la présente invention permet de transférer et d'exprimer un gène dans les cellules endothéliales de l'oeil.

- Injections intravitreuses

Des injections intravitreuses ont également été réalisées, dans le but d'infecter différents types de cellules de la rétine. Contrairement à la distribution uniforme dans les cellules endothéliales après injection au niveau de l'espace chambre anterieure, la distribution des cellules positives (infectées) après injection intravitreuse est limitée à l'hémirétine correspondant au point d'injection. La taille importante du cristallin et les caractéristiques de viscosité de l'humeur vitrée pourraient expliquer cette expression

confinée. Cependant, lorsque des injections temporales et nasales sont effectuées simultanément, les cellules des 2 hémirétines sont infectées. Ces résultats montrent donc qu'il est possible de transférer et d'exprimer un gène au niveau de la rétine. Ils montrent également que, selon la pathologie à traiter, et notamment selon sa distribution sur la rétine, il est possible de cibler le transfert sur une hémirétine seulement.

Trois couches nucléaires, correspondant aux cellules ganglionaires, bipolaires et photoréceptrices présentent également une coloration intense à trois semaines (age à partir duquel le développement de la rétine est terminé), ainsi que chez les souris adulte. Malgré la présence du signal permettant la localisation nucléaire de la protéine LacZ, le marquage (et donc l'infection) de certaines cellules au niveau du site d'injection est si intense que la coloration diffuse dans le cytoplasme. Pour cette raison, le couche de fibre nerveuse correspondant aux axones des noyaux marqués (qui convergent pour former le nerf optique) est marquée de manière homogène.

Une analyse fine des différentes couches de cellules rétiniennes ne fait apparaitre aucune diminution significative de leur épaisseur. De plus, la tête du nerf optique n'est pas altérée, même à des doses élevées d'adénovirus (10^7 pfu).

- Injection au niveau de l'espace rétrobulbaire

Pour évaluer la possibilité d'une diffusion du virus à travers la sclera, des souris ont été injectées au niveau de l'espace rétrobulbaire. Contrairement à la coloration rétinienne, environ 100 % des fibres des 4 muscles oculomoteurs ont été infectées et expriment l'activité β-galactosidase.

L'ensemble de ces résultats démontre clairement que les adénovirus recombinants, défectifs pour la réplication, constituent des vecteurs particulièrement intéressants pour le transfert de gènes in vivo dans les cellules oculaires.

REVENDICATIONS

1. Utilisation d'un virus recombinant défectif contenant un gène inséré pour la préparation d'une composition pharmaceutique destinée au traitement des pathologies oculaires.

2. Utilisation selon la revendication 1 caractérisée en ce que le virus recombinant défectif est dépourvu des régions de son génome qui sont nécessaires à sa réplication dans la cellule infectée.

3. Utilisation selon les revendications 1 ou 2 caractérisée en ce que le virus recombinant défectif est un adénovirus.

4. Utilisation selon la revendication 3 caractérisée en ce que l'adénovirus recombinant défectif est un adénovirus de type Ad 5.

5. Utilisation selon l'une des revendications 1 à 4 caractérisée en ce que le gène inséré comprend des séquences permettant son expression dans la cellule infectée.

6. Utilisation selon l'une des revendications 1 à 5 caractérisée en ce que le gène inséré code pour une protéine ou un fragment de protéine.

7. Utilisation selon l'une des revendications 1 à 5 caractérisée en ce que le gène inséré est une séquence antisens.

8. Utilisation selon la revendication 1 pour la préparation d'une composition pharmaceutique destinée au traitement des pathologies héréditaires telles que les rétinites pigmentaires.

9. Composition pharmaceutique comprenant une quantité suffisante de virus recombinant défectif selon la revendication 1, sous une forme adaptée à un usage oculaire.

10. Composition pharmaceutique selon la revendication 9 caractérisée en ce qu'elle comprend une quantité suffisante de virus recombinant défectif dans une forme injectable adaptée à un usage oculaire.

11. Composition pharmaceutique selon la revendication 9 caractérisée en ce qu'elle comprend une quantité suffisante de virus recombinant défectif sous une forme de collyre ou de pommade ophtalmique adaptés à un usage oculaire.

12. Composition pharmaceutique selon l'une des revendications 9 à 11 caractérisée en ce que le virus recombinant défectif est un adénovirus recombinant défectif.

13. Composition pharmaceutique selon la revendication 12 caractérisée en ce qu'elle comprend entre 10^4 et 10^{14} pfu/ml, et de préférence 10^6 à 10^{10} pfu/ml d'adénovirus recombinant défectif.

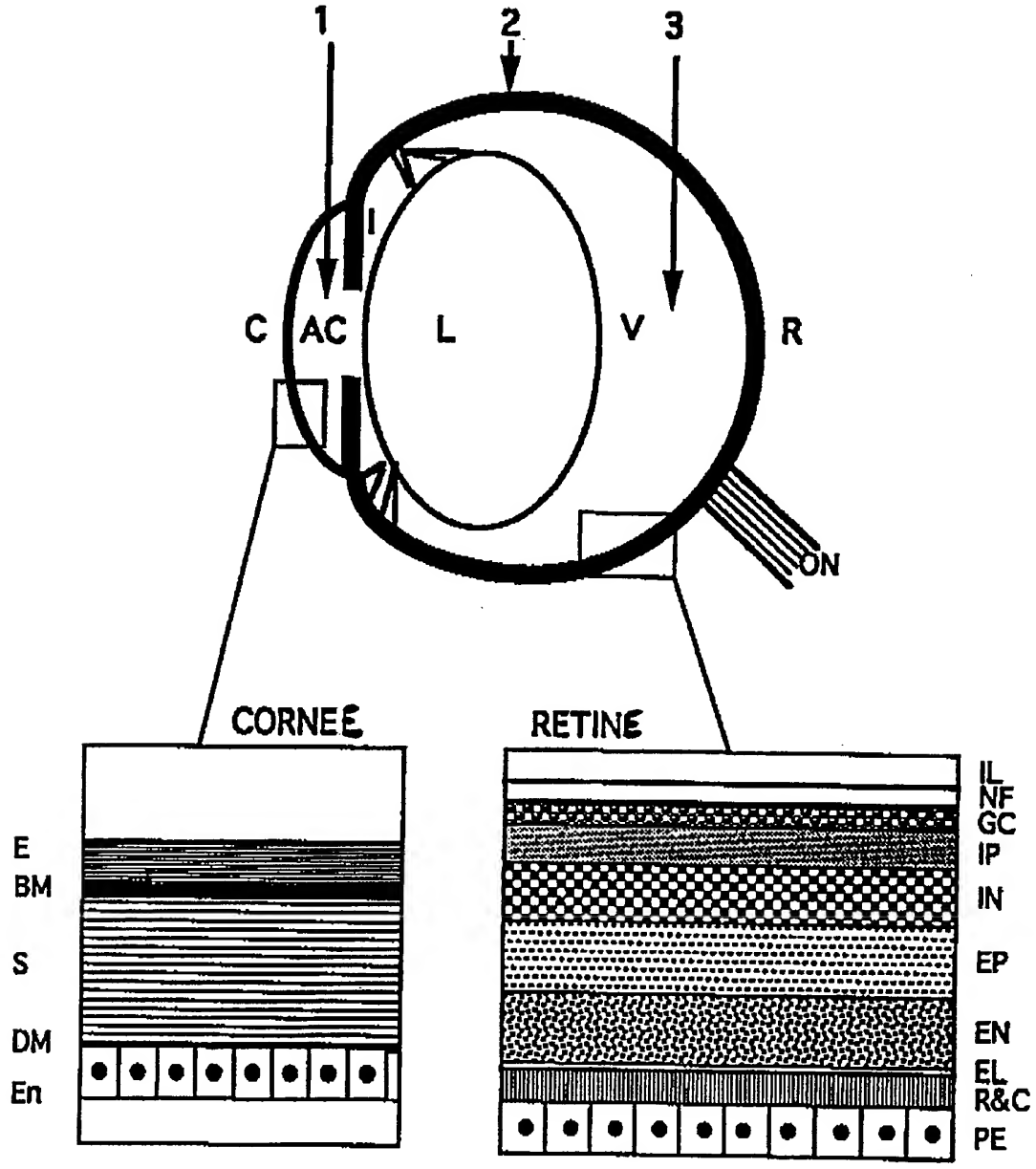
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FIGURE 1

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RAPPORT DE RECHERCHE

PRELIMINAIRE

établi sur la base des dernières revendications déposées avant le commencement de la recherche

FA 482236
FR 9302438

DOCUMENTS CONSIDERES COMME PERTINENTS

Catégorie	Citation du document avec indication, en cas de besoin, des parties pertinentes	Revendications concernées de la demande examinée
X	INVESTIGATIVE OPHTHALMOLOGY & VISUAL SCIENCE vol. 34, no. 3 , Mars 1993 pages 473 - 476 D. BOK 'Retinal transplantation and gene therapy'	1,2,5,6, 8-11
Y	* le document en entier *	3,4,7, 12,13

X	WO-A-90 02551 (BIOSOURCE GENETICS CORPORATION) 22 Mars 1990	1,2,5,6, 8-11
Y	* page 13, alinéa 1-2 * * page 19 * * page 35 - page 36; revendications 19,20 *	3,4,7, 12,13

Y	EXPERIMENTAL EYE RESEARCH vol. 50, no. 5 , Mai 1990 pages 521 - 532 L. STRAMM ET AL 'beta-Glucuronidase mediated pathway essential for retinal pigment epithelial degradation of glycosaminoglucans' * le document en entier *	3-13

X	THE NEW BIOLOGIST vol. 3, no. 3 , Mars 1991 pages 203 - 218 X. BREAKEFIELD AND N. DELUCA 'Herpes simplex virus for gene delivery to neurons'	1,2
Y	* le document en entier *	3-13

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DOMAINES TECHNIQUES RECHERCHES (Int.Cl.5)

C12N

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Date d'achèvement de la recherche: 1 Décembre 1993

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DOCUMENTS CONSIDERES COMME PERTINENTS

Catégorie	Citation du document avec indication, en cas de besoin, des parties pertinentes	Revendications concernées de la demande examinée
X	SCIENCE. vol. 259 , 12 Février 1993 , LANCASTER, PA US pages 988 - 990 G. LE GAL LA SALLE ET AL 'An adenovirus vector for gene transfer into neurons and glia in the brain' * le document en entier *	1-6, 8-10,12, 13
Y		7,11
Y	WO-A-92 17211 (EDISON ANIMAL BIOTECHNOLOGY CENTER) 15 Octobre 1992 * abrégé; revendications *	7
X	BONE MARROW TRANSPLANTATION vol. 9, no. SUP1 , 1992 pages 151 - 152 L. STRATFORD-PERRICAUDET ET AL 'Feasibility of adenovirus-mediated gene transfer in vivo' * le document en entier *	1-6, 8-10,12, 13
X	HUMAN GENE TRANSFER vol. 219 , 1991 pages 51 - 61 L. STRATFORD-PERRICAUDET AND M. PERRICAUDET 'Gene transfer into animals: the promise of adenovirus' * le document en entier *	1-6, 8-10,12, 13

DOMAINES TECHNIQUES RECHERCHES (Int.Cl.5)

Date d'achèvement de la recherche: 1 Décembre 1993

Examinateur: Van der Schaal, C

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Human Mol. Genet. 1(9):769-71, 1992.

Nucleic Acids Res. 19(24):6982 to end of article, 1991.

Investigative Opthalmology and Visual Science 36(4):ps1045, 1995, please attach date of availablity of this volume and number of journal.

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American Journal of Human genetics 55 (3 Suppl.):pA358, 1994.

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(54) Titre: ADENOVIRUS RECOMBINANTS ET LEUR UTILISATION EN THERAPIE GENIQUE POUR LE TRAITEMENT DES PATHOLOGIES OCULAIRES

(57) Abstract

The use of defective recombinant adenoviruses containing an inserted gene for preparing a pharmaceutical useful for treating ye diseases is disclosed.

(57) Abrégé

La présente invention concerne l'utilisation d'adénovirus recombinants défectifs contenant un gène inséré pour la préparation d'une composition pharmaceutique destinée au traitement des pathologies oculaires.

UNIQUEMENT A TITRE D'INFORMATION

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ADENOVIRUS RECOMBINANTS ET LEUR UTILISATION EN THERAPIE GENIQUE POUR LE TRAITEMENT DES PATHOLOGIES OCULAIRES

La présente invention concerne de nouveaux virus recombinants, leur préparation et leur utilisation en thérapie génique, pour le transfert et l'expression de gènes au niveau de l'oeil. Elle concerne également des compositions pharmaceutiques comprenant lesdits virus recombinants. Plus particulièrement, la présente invention concerne des virus recombinants défectifs et leur utilisation pour le traitement de pathologies oculaires.

Le traitement des pathologies oculaires, et notamment des maladies héréditaires constitue un problème non résolu actuellement. Parmi ces pathologies, on peut citer par exemple les rétinites pigmentaires, qui résultent d'altérations génétiques, et pour lesquelles aucun traitement n'est actuellement disponible. Par ailleurs, les pathologies non héréditaires telles que les atteintes post-inflammatoires (dégénérescence rétinienne, etc) ne disposent pas non plus aujourd'hui de traitement adapté. En particulier, si l'on tente d'agir préventivement, notamment au moyen de corticoïdes, on ne dispose actuellement d'aucun moyen satisfaisant pour traiter ces atteintes.

Il est donc important de pouvoir disposer d'outils permettant un traitement spécifique, efficace et localisé des pathologies oculaires. La présente invention apporte une solution avantageuse à ce problème, en démontrant la possibilité de traiter les pathologies oculaires par la thérapie génique.

La thérapie génique consiste à corriger une déficience ou une anormalité (mutation, expression aberrante, etc) par introduction d'une information génétique dans la cellule ou l'organe affecté. Cette information génétique peut être introduite soit in vitro dans une cellule extraite de l'organe, la cellule modifiée étant alors réintroduite dans l'organisme, soit directement in vivo dans le tissu approprié. Dans ce second cas, différentes techniques existent, parmi lesquelles des techniques diverses de transfection impliquant des complexes d'ADN et de DEAE-dextran (Pagano et al., J.Virol. 1 (1967) 891), d'ADN et de protéines nucléaires (Kaneda et al., Science 243 (1989) 375), d'ADN et de lipides (Felgner et al., PNAS 84 (1987) 7413), l'emploi de liposomes (Fraley et al., J.Biol.Chem. 255 (1980) 10431), etc. Plus récemment, l'emploi de virus comme vecteurs pour le transfert de gènes est apparu comme une alternative prometteuse à ces techniques physiques de transfection. A cet égard,

différents virus ont été testés pour leur capacité à infecter certaines populations cellulaires. En particulier, les rétrovirus (RSV, HMS, MMS, etc), le virus HSV, les virus adéno-associés, et les adénovirus.

Toutefois, jusqu'à présent, aucun de ces vecteurs n'a été utilisé ni décrit comme utilisable pour le transfert de gènes au niveau de l'oeil. La présente invention constitue la première démonstration qu'il est possible de traiter les pathologies oculaires par la thérapie génique.

Un premier objet de l'invention réside dans l'utilisation d'un virus recombinant défectif contenant un gène inséré pour la préparation d'une composition pharmaceutique destinée au traitement des pathologies oculaires.

Plus particulièrement, on utilise selon la présente invention des virus recombinants défectifs dérivés de virus capables d'infecter et d'exprimer un gène inséré dans les cellules de l'oeil, sans entraîner des phénomènes cytopathologiques ou d'effets pathogènes. Des virus susceptibles d'être utilisés dans l'invention sont par exemple les adénovirus, les virus adéno-associés ou encore le virus HSV.

La présente invention repose plus particulièrement sur la mise en évidence que les virus de type adénovirus sont capables de transférer et d'exprimer des gènes désirés au niveau de l'oeil. Les exemples présentés plus loin montrent que les adénovirus sont capables, selon le mode d'administration, de transférer efficacement, pour une durée importante et sans effet cytopathologique, des gènes dans l'endothélium cornéen, dans les cellules photoréceptrices, dans les cellules du nerf optique, dans les cellules bipolaires, etc. Par ailleurs, compte tenu de l'accès relativemant aisé aux différents compartiments de l'oeil par la microchirurgie (microinjection), ainsi que de l'existence de barrières naturelles dans cet organe (membrane de Descemet, membrane de Bruch, cristallin, etc) la présente invention permet avantageusement d'effectuer un transfert de gènes très ciblé, en fonction de la pathologie à traiter. Les résultats présentés montrent également que l'expression d'un gène désiré est stable sur une longue période (aucune perte d'activité à 50 jours).

Dans un mode de réalisation préféré, l'invention réside dans l'utilisation d'un adénovirus recombinant défectif contenant un gène inséré pour la préparation d'une composition pharmaceutique destinée au traitement des pathologies oculaires.

Le terme "virus ou adénovirus défectif" désigne un virus incapable de se répliquer de façon autonome dans la cellule cible. Généralement, le génome des virus

défectifs utilisés dans le cadre de la présente invention est donc dépourvu au moins des séquences nécessaires à la réplication dudit virus dans la cellule infectée. Ces régions peuvent être soit éliminées (en tout ou en partie), soit rendues non-fonctionnelles, soit substituées par d'autres séquences et notamment par le gène inséré. Préférentiellement, le virus défectif conserve néanmoins les séquences de son génome qui sont nécessaires à l'encapsidation des particules virales.

S'agissant plus particulièrement des adénovirus, il en existe différents sérotypes, dont la structure et les propriétés varient quelque peu. Néanmoins, ces virus ne sont pas pathogènes pour l'homme, et notamment les sujets non immuno-déprimés. Parmi ces sérotypes, on préfère utiliser dans le cadre de la présente invention les adénovirus de type 2 ou 5 (Ad 2 ou Ad 5). Dans le cas des adénovirus Ad 5, les séquences nécessaires à la réplication sont les régions E1A et E1B.

Des virus recombinants défectifs dérivés de rétrovirus, de virus adéno-associés ou du virus HSV (herpes simplex virus) ont déjà été décrits dans la littérature [Roemer et Friedmann, Eur. J. Biochem. 208 (1992) 211 ; Dobson et al., Neuron 5 (1990) 353 ; Chiocca et al., New Biol. 2 (1990) 739 ; Miyanohara et al., New Biol. 4 (1992) 238; WO91/18088].

Au sens de la présente invention, le terme "gène inséré" désigne toute séquence d'ADN introduite dans le virus recombinant, dont l'expression dans la cellule cible est recherchée.

Il peut s'agir en particulier d'un (ou plusieurs) gène(s) de structure codant pour une (des) protéine(s) ou pour une partie d'une (de) protéine(s). La protéine ou partie de protéine ainsi codée peut être une protéine homologue vis-à-vis de la cellule cible (c'est-à-dire une protéine qui est normalement exprimée dans la cellule cible lorsque celle-ci ne présente aucune pathologie), ou une protéine hétérologue vis-à-vis de ladite cellule. Dans le premier cas, l'expression de la protéine permet par exemple de pallier une expression insuffisante dans la cellule ou l'expression d'une protéine inactive ou faiblement active en raison d'une modification, ou encore de surexprimer ladite protéine. Dans le second cas, la protéine exprimée peut par exemple compléter ou apporter une activité déficiente dans la cellule lui permettant de lutter contre une pathologie.

Parmi les gènes insérés au sens de la présente invention, on peut citer plus particulièrement :

- les gènes impliqués dans des pathologies génétiques oculaires,

- les gènes codant pour des facteurs de croissance, des cytokines ou des neurotrophines : Le role protecteur ou curatif du produit d'expression de ces gènes dans différentes pathologies oculaires a été démontré, et notamment sur la détérioration des cellules photoréceptrices sous l'effet de la lumière (Lavail et al., PNAS 89 (1992) 11249).

- les gènes de facteurs de régulation (facteurs de transcription, facteurs de traduction),

- les gènes codant pour des enzymes,

- les gènes codant pour des protéines ayant des propriétés anticancéreuses, telles que les interférons, les facteurs de nécrose des tumeurs, etc, ou encore,

- les gènes codant pour des antigènes permettant une vaccination (une protection) locale contre une infection de l'oeil.

A titre d'exemples spécifiques, mais non limitatifs, on peut citer :

. le gène de l'ornithine aminotransférase impliqué dans l'atrophie gyrée (Akaki et al., J. Biol. Chem. 267(18) (1992) 12950),

. le gène de la rhodopsine, impliqué dans une forme de rétinite pigmentaire (Dryja et al., Nature 343 (1990) 364),

. le gène de la périphérine RDS, impliqué dans une forme de rétinite pigmentaire (Farrar et al., Nature 354 (1991) 478),

. le gène de la tyrosinase, impliqué dans l'albinisme oculocutanée type B1 (Giebel et al., Am. J. Hum. Genet. 48 (1991) 1159),

. le gène mitochondrial NDI, impliqué dans la maladie de Leber (Howell et al., Am. J. Hum. Genet. 48 (1991) 935),

. le gène de la sous-unité β de la cGMP phosphodiestérase, qui permet de ralentir la dégénérescence rétinienne (Lem et al., PNAS 89 (1992) 4422),

. le gène de la rab géranylgéranyl transférase, dont la déficience semble liée à une dégénérescence rétinienne lors de choroïdermies (Seabra et al., Science 259 (1993) 377),

. le gène du facteur de croissance des fibroblastes basique (bFGF), capable de retarder la dégénérescence des cellules photoréceptrices observée dans certaines dystrophies rétiniennes héréditaires (Faktorovich et al., Nature 347 (1990) 83),

. le gène de l'interleukine-8, qui permet d'induire une néovascularisation dans la cornée (Strieter et al., Am. J. Pathol. 141(6) (1992) 1279).

Le terme "gène inséré" désigne également des séquences antisens, dont l'expression dans la cellule cible permet de contrôler l'expression de gènes ou la transcription d'ARNm cellulaires. De telles séquences peuvent par exemple être transcrites, dans la cellule cible, en ARN complémentaires d'ARNm cellulaires et bloquer ainsi leur traduction en protéine.

Généralement, le gène inséré comprend également des séquences permettant son expression dans la cellule infectée. Il peut s'agir des séquences qui sont naturellement responsables de l'expression dudit gène lorsque ces séquences sont susceptibles de fonctionner dans la cellule infectée. Il peut également s'agir de séquences d'origine différente (responsables de l'expression d'autres protéines, ou même synthétiques). Notamment, il peut s'agir de séquences issues du génome de la cellule que l'on désire infecter, ou du génome du virus utilisé. S'agissant d'adénovirus, on peut citer par exemple les promoteurs des gènes E1A, MLP, etc. En outre, ces séquences d'expression peuvent être modifiées par addition de séquences d'activation, de régulation, etc. Par ailleurs, lorsque le gène inséré ne comporte pas de séquences d'expression, il peut être inséré dans le génome du virus défectif en aval d'une telle séquence.

Dans ce qui suit sont décrites plus en détail la construction et l'utilisation d'adénovirus recombinants défectifs. Il est entendu néanmoins que cette description peut être appliquée par l'homme du métier aux autres virus suceptibles d'être utilisés dans le cadre de la présente invention, ainsi qu'indiqués plus haut.

Les adénovirus recombinants défectifs peuvent être préparés par recombinaison homologue entre un adénovirus et un plasmide portant entre autre le gène que l'on désire insérer. La recombinaison homologue se produit après co-transfection desdits adénovirus et plasmide dans une lignée cellulaire appropriée. La lignée cellulaire utilisée doit de préférence (i) être transformable par lesdits éléments, et (ii), comporter les séquences capables de complémenter la partie du génome de l'adénovirus défectif, de préférence sous forme intégrée pour éviter les risques de recombinaison. A titre d'exemple de lignée, on peut mentionner la lignée de rein embryonnaire humain 293 (Graham et al., J. Gen. Virol. 36 (1977) 59) qui contient notamment, intégrée dans son génome, la partie gauche du génome d'un adénovirus Ad5 (12 %).

Ensuite, les vecteurs qui se sont multipliés sont récupérés et purifiés selon les techniques classiques de biologie moléculaire.

La présente invention concerne également une composition pharmaceutique comprenant une quantité suffisante de virus recombinant défectif tel que décrit précédemment, sous une forme adaptée à un usage oculaire.

En particulier, le virus recombinant défectif peut être sous forme de solution injectable, de collyre, de pommade ophtalmique, etc. Les véhicules pharmaceutiquement acceptables pour de telles formulations adaptées à un usage oculaire sont notamment des solutions salines (phosphate monosodique, disodique, clrorure de sodium, potassium, calcium ou magnésium, etc, ou des mélanges de tels sels), la vaseline, l'huile de vaseline, etc.

Dans le cas de collyres ou de pommades ophtalmiques, il est entendu que les applications thérapeutiques peuvent être plus limitées en raison d'une diffusion plus faible du virus recombinant défectif.

Dans leur utilisation pour le traitement des pathologies oculaires, les virus recombinants défectifs selon l'invention peuvent être administrés selon différents modes, et notamment par injection sousrétinale, éventuellement précédée d'une vitrectomie, ou par injection intravitreuse, simples ou multiples (voir figure 1). L'injection sous-rétinale peut être réalisée sélectivement dans différents compartiments de l'oeil, et notamment, l'injection peut être réalisée au niveau du vitré, de la chambre anterieure ou de l'espace rétrobulbaire. Les résultats présentés dans la présente demande montrent que ces différents modes d'injection permettent d'infecter de manière ciblée les différents tissus de l'oeil, et notamment, l'endothélium cornéen, les cellules photoréceptrices, les cellules bipolaires, les cellules ganglionaires ou encore les cellules des muscles oculomoteurs.

Les doses de virus utilisées pour l'injection peuvent être adaptées en fonction de différents paramètres, et notamment en fonction du mode d'administration utilisé, de la pathologie concernée, du gène à exprimer, ou encore de la durée du traitement recherchée. D'une manière générale, les adénovirus recombinants selon l'invention sont formulés et administrés sous forme de doses comprises entre 10^4 et 10^{14} pfu/ml, et de préférence 10^6 à 10^{10} pfu/ml. Le terme pfu ("plaque forming unit") correspond au pouvoir infectieux d'une solution de virus, et est déterminé par infection d'une culture cellulaire appropriée, et mesure, généralement après 48 heures, du nombre de plages

de cellules infectées. Les techniques de détermination du titre pfu d'une solution virale sont bien documentées dans la littérature.

Compte tenu de la stabilité d'expression du gène inséré dans la cellule cible, la présente invention devrait permettre de traiter la majorité des pathologies oculaires avec peu d'injections.

La présente invention offre ainsi un moyen très efficace pour le traitement des pathologies oculaires, et notamment celles dont les mécanismes ont été élucidés au niveau moléculaire. En particulier, l'implication de gènes a été démontrée dans l'atrophie gyrée, dans la maladie de Norrie (Hum. Mol. Genet. 1(7) (1992) 461), dans la dégénérescence rétinienne (Bowes et al., PNAS 86 (1989) 9722), dans la maladie de Leber, dans les choroïdermies (Cremers et al., Nature 347 (1990) 674), dans la dégénérescence des cellules photoréceptrices, dans les rétinites pigmentaires, dans l'albinisme, dans le syndrome Kearns-Sayre (Shoffner et al., PNAS 86 (1989) 7952), etc. La présente invention est également pour le traitement des altérations de la cornée acquises résultant de maladies inflammatoires, des atteintes rétiniennes post-inflammatoires, etc.

La présente invention rend également possible la thérapie par les protéines ou peptides, dont l'utilisation par les voies classiques d'administration est très hypothétique en raison de leur forte sensibilité aux mécanismes de dégradation et d'élimination de l'organisme, et des problèmes liés à la pénétration dans les cellules. L'emploi de virus selon l'invention permet d'exprimer directement à l'interieur de la population de cellules ciblées le polypeptide ou la protéine désirée, qui n'est donc plus accessible aux mécanismes mentionnés ci-avant.

L'ensemble des résultats présentés dans la présente demande démontre plus particulièrement que les adénovirus recombinants, défectifs pour la réplication, constituent des vecteurs particulièrement intéressants pour le transfert de gènes in vivo dans les cellules oculaires. Les expériences réalisées montrent la possibilité d'une expression stable à long-terme de gènes dans ces cellules. En particulier, une expression stable est observée 50 jours après l'injection. De plus, le spectre d'expression large dans les différentes cellules oculaires constitue également un résultat particulièrement intéressant dans la mesure où pratiquement toutes les maladies de la rétine (notamment la retinitis pigmentosa) affectent une grande surface de la rétine.

En outre, ce traitement peut concerner aussi bien l'homme que tout animal tel que les ovins, les bovins, les animaux domestiques (chiens, chats, etc), les chevaux, les poissons, etc.

La présente invention est plus complètement décrite à l'aide des exemples qui suivent, qui doivent être considérés comme illustratifs et non limitatifs.

Légende des figures

Figure 1 : Représentation schématique de l'oeil. C = cornée; AC = Chambre antérieure; L = cristallin; V = vitré; I = iris; ON = nerf optique; R = espace rétrobulbaire.

Construction d'un adénovirus recombinant défectif (Ad.RSVβGal) :

La procédure générale permettant la préparation des adénovirus recombinants a été décrite dans la partie générale de la description.

L'adénovirus Ad.RSVβGal est un adénovirus recombinant défectif (délété des régions E1 et E3) obtenu par recombinaison homologue in vivo entre l'adénovirus mutant Ad-d1324 (Thimmappaya et al., Cell 31 (1982) 543) et le plasmide pAd.RSVβGal (Akli et al. 1993).

Le plasmide pAd.RSVβGal contient, dans l'orientation 5'->3',

- le fragment PvuII correspondant à l'extrémité gauche de l'adénovirus Ad5 comprenant : la séquence ITR, l'origine de réplication, les signaux d'encapsidation et l'amplificateur E1A;
- le gène codant pour la β-galactosidase sous le contrôle du promoteur RSV (du virus du sarcome de Rous),
- un second fragment du génome de l'adénovirus Ad5, qui permet la recombinaison homologue entre le plasmide pAd.RSVβGal et l'adénovirus d1324.

Après linéarisation par l'enzyme ClaI, le plasmide pAd.RSVβGal et l'adénovirus d1324 sont co-transfectés dans la lignée 293 en présence de phosphate de calcium pour permettre la recombinaison homologue. Les adénovirus recombinants ainsi générés sont sélectionnés par purification sur plaque. Après isolement, l'ADN de l'adénovirus recombinant est amplifié dans la lignée cellulaire 293, ce qui conduit à un surnageant de culture contenant l'adénovirus défectif recombinant non purifié ayant un titre d'environ 10^{10} pfu/ml.

Les particules virales sont généralement purifiées par centrifugation sur gradient de chlorure de césium selon les techniques connues (voir notamment Graham et al., Virology 52 (1973) 456). L'adénovirus Ad.RSVβGal est conservé à -80°C dans 20 % de glycérol. Avant injection, la suspension d'adénovirus est diluée au tiers dans un tampon phosphate PBS.

Injection in vivo

- Protocole

Des souris C57Bl/6 de 3 à 7 semaines ont été anesthésiées avec de l'avertine. Dans chaque oeil a ensuite été injecté 10^7 à 10^8 pfu d'adénovirus recombinant Ad.RSVβGal, soit au niveau de la chambre anterieure, soit au niveau du vitré, soit au niveau de l'espace rétrobulbaire (voir figure 1). Les animaux ont été sacrifiés 7 à 50 jours après l'injection par dislocation cervicale et les yeux ont été récupérés et fixés dans l'azote liquide. Des sections sagitales et coronales (10-15 µm) sont réalisées sur cryostat, puis colorées en présence de X-gal pour révéler l'activité β-galactosidase qui peut être visualisée par l'apparition d'une coloration bleue dans le noyau des cellules infectées, et contre-colorées avec de l'hémotoxyline et de l'éosine.

- Injection au niveau de la chambre anterieure

Après injection de 10^8 pfu d'adénovirus Ad.RSVβGal au niveau de l'espace de la chambre anterieure, seules les cellules de la couche endotheliale expriment l'activité β-galactosidase. En revanche, les cellules épithéliales ou du stroma ne présentent aucune coloration à la suite d'une telle injection. De plus, les cellules marquées (infectées) sont distribuées régulièrement dans la couche endothéliale, quel que soit le temps d'administration. Ce résultat montre que la présente invention permet de transférer et d'exprimer un gène dans les cellules endothéliales de l'oeil.

- Injections intravitreuses

Des injections intravitreuses ont également été réalisées, dans le but d'infecter différents types de cellules de la rétine. Contrairement à la distribution uniforme dans les cellules endothéliales après injection au niveau de l'espace chambre anterieure, la distribution des cellules positives (infectées) après injection intravitreuse est limitée à l'hémirétine correspondant au point d'injection. La taille importante du cristallin et les caractéristiques de viscosité de l'humeur vitrée pourraient expliquer cette expression

confinée. Cependant, lorsque des injections temporales et nasales sont effectuées simultanément, les cellules des 2 hémirétines sont infectées. Ces résultats montrent donc qu'il est possible de transférer et d'exprimer un gène au niveau de la rétine. Ils montrent également que, selon la pathologie à traiter, et notamment selon sa distribution sur la rétine, il est possible de cibler le transfert sur une hémirétine seulement.

Trois couches nucléaires, correspondant aux cellules ganglionaires, bipolaires et photoréceptrices présentent également une coloration intense à trois semaines (age à partir duquel le développement de la rétine est terminé), ainsi que chez les souris adulte. Malgré la présence du signal permettant la localisation nucléaire de la protéine LacZ, le marquage (et donc l'infection) de certaines cellules au niveau du site d'injection est si intense que la coloration diffuse dans le cytoplasme. Pour cette raison, le couche de fibre nerveuse correspondant aux axones des noyaux marqués (qui convergent pour former le nerf optique) est marquée de manière homogène.

Une analyse fine des différentes couches de cellules rétiniennes ne fait apparaitre aucune diminution significative de leur épaisseur. De plus, la tête du nerf optique n'est pas altérée, même à des doses élevées d'adénovirus (10^7 pfu).

- Injection au niveau de l'espace rétrobulbaire

Pour évaluer la possibilité d'une diffusion du virus à travers la sclera, des souris ont été injectées au niveau de l'espace rétrobulbaire. Contrairement à la coloration rétinienne, environ 100 % des fibres des 4 muscles oculomoteurs ont été infectées et expriment l'activité β-galactosidase.

L'ensemble de ces résultats démontre clairement que les adénovirus recombinants, défectifs pour la réplication, constituent des vecteurs particulièrement intéressants pour le transfert de gènes in vivo dans les cellules oculaires.

REVENDICATIONS

1. Utilisation d'un adénovirus recombinant défectif contenant un gène inséré pour la préparation d'une composition pharmaceutique destinée au traitement des pathologies oculaires.

2. Utilisation selon la revendication 1 caractérisée en ce que l'adénovirus recombinant défectif est dépourvu des régions de son génome qui sont nécessaires à sa réplication dans la cellule infectée.

3. Utilisation selon les revendications 1 ou 2 caractérisée en ce que l'adénovirus recombinant défectif est un adénovirus de type Ad 2.

4. Utilisation selon la revendication 1 ou 2 caractérisée en ce que l'adénovirus recombinant défectif est un adénovirus de type Ad 5.

5. Utilisation selon l'une des revendications 1 à 4 caractérisée en ce que le gène inséré comprend des séquences permettant son expression dans la cellule infectée.

6. Utilisation selon l'une des revendications 1 à 5 caractérisée en ce que le gène inséré code pour une protéine ou un fragment de protéine.

7. Utilisation selon l'une des revendications 1 à 5 caractérisée en ce que le gène inséré est une séquence antisens.

8. Utilisation selon la revendication 1 pour la préparation d'une composition pharmaceutique destinée au traitement des pathologies héréditaires telles que les rétinites pigmentaires.

9. Composition pharmaceutique comprenant une quantité suffisante d'adénovirus recombinant défectif selon la revendication 1, sous une forme adaptée à un usage oculaire.

10. Composition pharmaceutique selon la revendication 9 caractérisée en ce qu'elle comprend une quantité suffisante d'adénovirus recombinant défectif dans une forme injectable adaptée à un usage oculaire.

11. Composition pharmaceutique selon la revendication 9 caractérisée en ce qu'elle comprend une quantité suffisante d'adénovirus recombinant défectif sous une forme de collyre ou de pommade ophtalmique adaptés à un usage oculaire.

12. Composition pharmaceutique selon l'une des revendications 9 à 11 caractérisée en ce que l'adénovirus recombinant défectif est un adénovirus recombinant défectif de type Ad2 ou Ad5.

13. Composition pharmaceutique selon la revendication 12 caractérisée en ce qu'elle comprend entre 10^4 et 10^{14} pfu/ml, et de préférence 10^6 à 10^{10} pfu/ml d'adénovirus recombinant défectif.

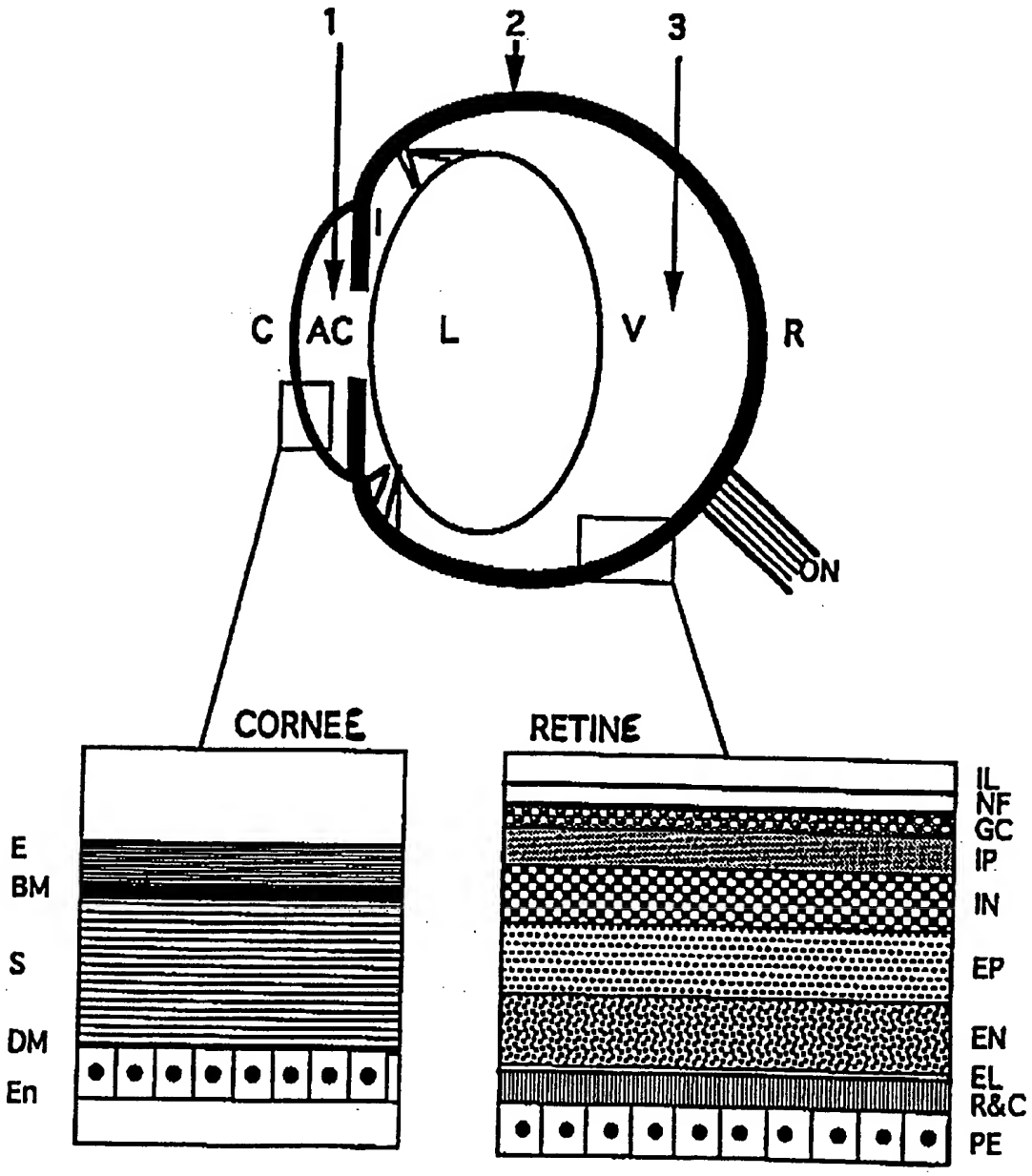
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FIGURE 1

INTERNATIONAL SEARCH REPORT

Int onal Application No
PCT/FR 94/00220

A. CLASSIFICATI N OF SUBJECT MATTER
IPC 5 A61K48/00 C12N15/86 //C12N7/01

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,90 02551 (BIOSOURCE GENETICS CORPORATION) 22 March 1990 see page 13, paragraph 1-2 see page 19 see page 36, paragraph 2-3; claims 19,20 ---	1-13
Y	THE NEW BIOLOGIST vol. 3, no. 3 , March 1991 pages 203 - 218 X. BREAKEFIELD AND N. DELUCA 'Herpes simplex virus for gene delivery to neurons' see the whole document --- -/--	1-13

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

2

Date of the actual completion of the international search	Date of mailing of the international search report
9 June 1994	

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Van der Schaal, C

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/FR 94/00220

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EXPERIMENTAL EYE RESEARCH vol. 50, no. 5 , May 1990 pages 521 - 532 L. STRAMM ET AL 'beta-Glucuronidase mediated pathway essential for retinal pigment epithelial degradation of glycosaminoglucans' see the whole document	1-13
Y	SCIENCE. vol. 259 , 12 February 1993 , LANCASTER, PA US pages 988 - 990 G. LE GAL LA SALLE ET AL 'An adenovirus vector for gene transfer into neurons and glia in the brain' see the whole document	1-13
Y	BONE MARROW TRANSPLANTATION vol. 9, no. SUP1 , 1992 pages 151 - 152 L. STRATFORD-PERRICAUDET ET AL 'Feasibility of adenovirus-mediated gene transfer in vivo' see the whole document	1-13
Y	HUMAN GENE TRANSFER vol. 219 , 1991 pages 51 - 61 L. STRATFORD-PERRICAUDET AND M. PERRICAUDET 'Gene transfer into animals: the promise of adenovirus' see the whole document	1-13
Y	WO,A,92 17211 (EDISON ANIMAL BIOTECHNOLOGY CENTER) 15 October 1992 see abstract; claims	7
P,Y	INVESTIGATIVE OPHTHALMOLOGY & VISUAL SCIENCE vol. 34, no. 3 , 9 March 1993 pages 473 - 476 D. BOK 'Retinal transplantation and gene therapy' see the whole document	1-13

INTERNATI NAL SEARCH REPORT

Information on patent family members

Int .onal Application No
PCT/FR 94/00220

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9002551	22-03-90	AU-B-	633517	04-02-93
		AU-A-	4327089	02-04-90
		EP-A-	0434750	03-07-91
		JP-T-	4500672	06-02-92
		US-A-	5210076	11-05-93
WO-A-9217211	15-10-92	AU-A-	1778092	02-11-92
		CA-A-	2107789	06-10-92
		EP-A-	0578776	19-01-94

RAPPORT DE RECHERCHE INTERNATIONALE

De e Internationale No
PCT/FR 94/00220

A. CLASSEMENT DE L'OBJET DE LA DEMANDE
CIB 5 A61K48/00 C12N15/86 //C12N7/01

Selon la classification internationale des brevets (CIB) ou à la fois selon la classification nationale et la CIB

B. DOMAINES SUR LESQUELS LA RECHERCHE A PORTE

Documentation minimale consultée (système de classification suivi des symboles de classement)
CIB 5 C12N A61K

Documentation consultée autre que la documentation minimale dans la mesure où ces documents relèvent des domaines sur lesquels a porté la recherche

Base de données électronique consultée au cours de la recherche internationale (nom de la base de données, et si cela est réalisable, termes de recherche utilisés)

C. DOCUMENTS CONSIDERES COMME PERTINENTS

Catégorie *	Identification des documents cités, avec, le cas échéant, l'indication des passages pertinents	no. des revendications visées
Y	WO,A,90 02551 (BIOSOURCE GENETICS CORPORATION) 22 Mars 1990 voir page 13, alinéa 1-2 voir page 19 voir page 36, alinéa 2-3; revendications 19,20 ---	1-13
Y	THE NEW BIOLOGIST vol. 3, no. 3 , Mars 1991 pages 203 - 218 X. BREAKEFIELD AND N. DELUCA 'Herpes simplex virus for gene delivery to neurons' voir le document en entier --- -/--	1-13

[X] Voir la suite du cadre C pour la fin de la liste des documents

[X] Les documents de familles de brevets sont indiqués en annexe

* Catégories spéciales de documents cités:

"A" document définissant l'état général de la technique, non considéré comme particulièrement pertinent

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Date à laquelle la recherche internationale a été effectivement achevée	Date d'expédition du présent rapport de recherche internationale
9 Juin 1994	15 -06- 1994

Nom et adresse postale de l'administration chargée de la recherche internationale	Fonctionnaire autorisé
Office Européen des Brevets, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Van der Schaal, C

Formulaire PCT/ISA/210 (deuxième feuille) (juillet 1992)

RAPPORT DE RE[illegible]E INTERNATIONALE

Renseignements relatifs au[illegible] membres de familles de brevets

De[Int] le Internationale No
PCT/FR 94/00220

Document brevet cité au rapport de recherche	Date de publication	Membre(s) de la famille de brevet(s)		Date de publication
WO-A-9002551	22-03-90	AU-B-	633517	04-02-93
		AU-A-	4327089	02-04-90
		EP-A-	0434750	03-07-91
		JP-T-	4500672	06-02-92
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